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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.63(b))

Attorney Docket No. 960296.95912

First Inventor or Application Identifier Karen M. Downs

Title CHIMERIC MAMMALIAN ALLANTOIS

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APPLICATION ELEMENTS

See MPEP Chapter 600 concerning utility patent application contents.

ADDRESS TO:

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- 1 ☒ Fee transmittal Form
(Submit an original and a duplicate for fee processing)
- 2 ☒ Specification [Total Pages 116]
(preferred arrangement set forth below)
- Descriptive title of the invention
 - Cross References to Related Applications
 - Statement Regarding Fed Sponsored R&D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
- 3 ☒ Drawing(s) (35 USC 113) [Total Sheets 19]
4. Oath or Declaration [Total Pages 2]
- a. ☐ Newly executed (original or copy)
- b. ☐ Copy from prior Application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
- [Note Box 5 below]
- i. ☐ DELETION OF INVENTOR(S)
Signed Statement attached deleting
inventor(s) named in prior application,
see 37 CFR 1.63(d)(2) and 1.33(b).
- 5 ☐ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application from
which a copy of the oath or declaration is supplied
under Box 4b, is considered as being part of the
disclosure of the accompanying application and is
hereby incorporated by reference herein.

6. ☐ Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
- a. ☐ Computer readable Copy
- b. ☐ Paper Copy (identical to computer copy)
- c. ☐ Statement Verifying identity of above

ACCOMPANYING APPLICATION PARTS

- 8 ☐ Assignment Papers (cover sheet & documents)
- 9 ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(where there is an assignee)
- 10 ☐ English Translation Document (if applicable)
- 11 ☐ Information Disclosure
Statement (IDS)/PTO-1449 ☐ Copies of IDS
Citations
- 12 ☐ Preliminary Amendment
- 13 ☐ Return receipt postcard (MPEP 503)
(Should be specifically itemized)
- 14 ☐ *Small Entity ☐ Statement filed in prior application
Statement(s) ☐ Status still proper and desired
- 15 ☐ Certified copy of priority Document(s)
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- 16 ☐ Other:

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17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP) of prior application no. 08/838,384

Prior application information: Examiner: D. Clark Group/Art Unit: 1633

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CHIMERIC MAMMALIAN ALLANTOIS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Serial No. 08/838,384, filed April 8, 1997, U.S. Serial No. 60/015,066, filed April 9, 1996 and to U.S. Serial No. 60/118,764, filed February 5, 1999. Serial Nos. 60/015,066, 08/838,384 and 60/118,764 are herein incorporated by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND OF THE INVENTION

The major vascular systems of the developing fetus are formed by vasculogenesis, a developmental process in which mesoderm is transformed *in situ* into endothelial cells. The goal of my work is to discover how mesoderm is transformed into the endothelial cell lineage using the mouse allantois as a model *in vitro* system.

During its early development, the murine allantois consists of an inner core of mesoderm and an outer layer of squamous epithelium referred to as a mesothelium. The allantois undergoes two major developmental processes: (i) maturation and fusion with the chorion to become the umbilical component of the chorioallantoic placenta, and (ii) vascularization, forming an artery and a vein that permit within the chorionic disk the exchange of nutrients, metabolic wastes and gases with the mother

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during fetal gestation (K.M. Downs and R.L. Gardner, Development 121:407-416, 1995; K.M. Downs and C. Harmann, Development 124:2769-2780, 1997; K.M. Downs, et al., The Murine Allantois. In *Current Topics in Developmental Biology* (eds. R. Pedersen and G. Schatten). New York: Academic Press. 39:1-33, 1998; K.M. Downs, et al., Development, submitted, 1999; K.M. Downs, supra, 1998).

BRIEF SUMMARY OF THE INVENTION

In one embodiment, the present invention is a method
10 for evaluating the effect of test compounds, preferably
potentially harmful or beneficial substances, on
formation of blood vessels during vasculogenesis. In one
embodiment, this method comprises direct application of a
test compound to cultured allantoic explants. In another
15 embodiment, one would evaluate test compounds by DNA
uptake and expression of a test compound by the
mesenchymal cells of allantoic explants.

In another embodiment, the present invention is the
delivery of factors into the umbilical circulation by
20 formation of chimeric allantoises. Preferably, this
delivery ameliorates or eliminates developmental defects
through delivery of therapeutic factors.

In another embodiment, the present invention is the
delivery of factors into the umbilical circulation by
25 formation of chimeric allantoises. Preferably, this
delivery ameliorates or eliminates developmental defects

through delivery of therapeutic factors found in normal allantoic cells.

5 In another embodiment, the present invention is the delivery of gene expression products into the umbilical circulation by formation of a chimeric allantois. This method comprises the step of transfection of mesenchymal cells with heterologous genes expressed from appropriate endothelial cell promoters and introducing the donor mesenchymal cells into a developing allantois. Once
10 integrated into the vasculature, the cells will express the gene product and deliver it to the bloodstream of the fetus. Preferably, the gene product is a therapeutic protein targeted to a fetus with particular developmental defects.

15 In another embodiment, the present invention is a method of fetal gene therapy comprising the step of creating a chimeric allantois/umbilical cord. Specifically, the method involves identifying a fetus with a genetic defect and obtaining compatible
20 allantois/umbilical cells capable of expressing a gene product that would ameliorate this defect. The cells are introduced into the exocoelomic cavity of the defective embryo or transplanted into the conceptus and assimilated into the native allantois/umbilical cord. Thus, a
25 chimeric allantois/umbilical cord is produced. The gene product is then delivered into the fetus via the umbilical blood vessels. Preferably, the fetus is a

mammalian fetus. Most preferably, the fetus is a human fetus.

In another embodiment, the present invention is a method of delivering a heterologous protein to a fetus
5 via obtaining compatible allantois/umbilical cells capable of expressing a gene product and introducing these cells into the exocoelomic cavity of an embryo or transplanting the cells onto the embryo and assimilating them into the native allantois/umbilical cord. A
10 chimeric allantois/umbilical cord is produced and the gene product is then delivered into the fetus via the umbilical blood vessels.

In another embodiment, the present invention is a method of fetal gene therapy comprising the step of
15 creating a chimeric allantois/umbilical cord by obtaining allantois cells compatible with a fetus with a genetic defect that express a gene product that ameliorates the genetic defect. The cells are then transplanted into the fetus wherein the transplanted cells develop into
20 endothelial cells which line the vasculature of the umbilical cord and release the gene product into the bloodstream of the fetus.

The present invention is also a population of transgenic allantois cells, wherein the transgene may be
25 a therapeutic gene and/or a marker or reporter gene.

It is an object of the present invention to provide a method of human fetal gene therapy.

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It is another object of the present invention to provide a method of human fetal gene therapy that would benefit continued therapy after birth because the supplemented gene product would be recognized as "self" by the adult immune system.

Other advantages, objects and features of the present invention will be obvious after review of the specification, drawings and claims.

BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

10 Figs. 1A and B depict microsurgery of ROSA26/26 allantoises into genetically-similar non-transgenic hosts. Fig. 1A depicts the allantois of a ROSA26/26-expressing headfold-stage conceptus. Fig. 1B depicts injecting the allantois into the exocoelomic cavity of a
15 congenic non-transgenic host.

Fig. 2 is a schematic diagram of fetal gene therapy.

Fig. 3 is a schematic diagram of the allantois and delineation of the base at the level of insertion of the amnion.

20 Fig. 4A and B diagrams sites of transplantation.

Fig. 5 is a summary of results of grafts of donor allantoic tissue into the base of host allantoises, headfold stage.

25 Fig. 6 is a summary of results of grafts into prospective lateral plate mesoderm, headfold stage.

Fig. 7 is a summary of results of grafts into prospective paraxial mesoderm, headfold stage.

Fig. 8 diagrams colonization patterns by cell type of grafted allantoic tissue into host allantoises.

Fig. 9 is a "position-effect" model of angioblast formation in the allantois.

5 Fig. 10 describes the morphology and *lacZ* expression in plated headfold-stage allantoises. Figs. 10A-C describe the comparison of a single explanted allantois grown for 24 hours (Fig. 10A), 48 hours (Fig. 10B) and 72 hours (Fig. 10C) in a 24-well tissue culture plate
10 containing an equal volume of rat serum and DMEM. Figs. 10D-F show vascular channels, coalesced clusters of cells, and peripheral mesenchymal cells.

Fig. 11 describes the immunohistochemistry used to localize Flk-1 and VCAM-1 in plated headfold-stage
15 allantoises. Figs. 11A and B are low and high magnification of anti-Flk-1-stained allantoises. Figs. 11C and D are low and high magnification of anti-VCAM-1-stained allantoises. Figs. 11E and F are control allantoises minus primary antibody (Fig. 11E) and primary
20 antibody prebound with control peptide (Fig. 11F).

Fig. 12 illustrates the time course of vascularization in plated headfold-stage allantoises. Fig. 12A is at 4 hours, Fig. 12B is at 8 hours, Fig. 12C is at 12 hours, Fig. 12D is at 16 hours and Fig. 12E is
25 at 20 hours.

Fig. 13 describes transplantation of cultured donor *lacZ*/+ allantoic cells into cultured host conceptuses. Fig. 13A is a 8.75 dpc cultured benzidine-stained host

conceptus viewed from the region of the ectoplacental
cone to show the vessel-free region in the yolk sac
selected for injection of donor cells into the allantois.
Fig. 13B is a schematic diagram of cultured 8.75 dpc
5 conceptus in sagittal orientation showing the distal site
of injection of donor cells into the host allantois.
Fig. 13C is an operated 8.75 dpc host conceptus cultured
for a further 8 hours, viewed following removal of its
yolk sac. Fig. 13D is a schematic diagram of the fetus
10 in (13C) showing the regions of the allantois used to
score the location of blue donor cells. Fig. 13E
demonstrates cultured *lacZ*/+ blue donor cells are visible
in the distal and fusion junction regions of the host
allantois. (The insert is a higher magnification view of
15 donor cells in the chorioallantoic fusion junction
showing incorporation of donor cells in the host
endothelium.) Fig. 13F is a clump of unincorporated
donor cells in contact with, but not integrated into, the
host yolk sac.

20 Fig. 14 plots the average number of donor *lacZ*/+
allantoic cells incorporated into host allantoises
according to cell type.

Fig. 15 illustrates localization of Flk-1 in
allantoises of freshly-recovered (Fig. 15A), cultured
25 (Fig. 15B) and cultured chimeric (Fig. 15C) conceptuses.
Fig. 15D is a higher magnification view of doubly-stained
X-gal/Flk-1-positive donor cells.

Fig. 16 is a set of light- (Figs. 16A, C, E) and
fluorescent- (Figs. 16B, D, F) micrographs of E'oma cells
(Figs. 16A, B), L929 fibroblasts (Figs. 16C, D) and
headfold-stage allantois plated for 72 hours (Figs. 16E,
5 F).

Fig. 17 describes the spatiotemporal appearance of
VCAM-1 in freshly-recovered conceptuses. Fig. 17A is an
allantois at late headfold-stage (LHF). Fig. 17B is an
allantois at 4-somite pairs. Fig. 17C is a 6-somite pair
10 allantois. Fig. 17D is an 8-somite pair allantois. Fig.
17E is an 8-somite pair control conceptus without
antibody (-Ab). Fig. 17F is an 8-somite pair control
allantois in which antibody to VCAM-1 was prebound with
control VCAM-1 peptide (+cp).

Fig. 18 illustrates simultaneous localization of
15 Flk-1 and VCAM-1 in intact and suspended allantoises.
Fig. 18A is a freshly recovered conceptus containing 14-
somite pairs. Fig. 18B is a headfold-stage conceptus
cultured to 14-somite pairs. Fig. 18C is an explanted
20 headfold stage allantois cultured in suspension for 24
hours and doubly-immunostained.

Fig. 19 describes expression of GFP in plated
allantoic explants at low magnification (Fig. 19A)
fluorescence view, higher magnification (Fig. 19B) of
25 GFP-expressing mesenchymal cells from Fig. 19A, and
simultaneous bright field/fluorescent view (Fig. 19C) of
those GFP-expressing mesenchymal cells from Fig. 19B.

DETAILED DESCRIPTION OF THE INVENTION

1. Method of Delivering Therapeutic Molecules to a Fetus.

The present invention is a method of delivering
5 molecules, preferably therapeutic molecules, to a fetus.
In one embodiment, this method begins with identifying a
fetus with a genetic defect. Once one has identified
this defect, one then must obtain compatible allantois
cells capable of expressing a gene that would ameliorate
10 this defect. (These cells could be recombinant cells
expressing a foreign gene product or non-recombinant
cells expressing native gene products.) The Examples
below describe a proposed method of ameliorating
hemophilia A in transgenic mice defective in production
15 of Factor VIII with genetically-engineered cells which
can assimilate into the allantois and/or umbilical cord
to form a chimeric umbilical cord. Of course, other
genetic defects and diseases are known to one of skill in
the art of molecular biology.

20 We propose below to obtain compatible allantois or
umbilical cord cell lines that will then be manipulated
to express factor VIII. By "compatible," we mean cells
that may be assimilated into the native allantois to make
a chimeric allantois or umbilical cord. As described in
25 the Examples, whole allantoises from appropriate
conceptuses are removed with glass capillary pipettes and
placed in tissue culture dishes. When the explants are
fairly large, they will be disaggregated and passaged

into cells to reach confluence. These cells will be subcloned, transfected and tested for the appropriate gene activity. Alternatively, one might infect explants rather than cell lines and subclone after infection.

5 The present invention is also a transgenic allantois cell. The transgene may be a therapeutic gene or a marker gene.

 The Examples below demonstrate that cells taken from different areas of the allantois seem to have different
10 developmental potentials.

 In the allantois, the transplanted cells appeared as various cell types, including endothelial cells which would be expected to line the vasculature of the umbilical cord. Thus, these transplanted cells could
15 potentially be useful for releasing the products of transfected genes into the bloodstream of a developing fetus. Genetic engineering of these allantoic cells could also be useful for studying the function of various genes in normal vasculogenesis and in malfunctions of
20 vasculogenesis.

 It may therefore be advantageous in some applications to obtain cells from only one portion of the allantois and manipulate and transplant these specially selected cells. For example, the examples below describe
25 the appearance of transgenic endothelial cells after transplantation of transgenic allantois cells onto a non-transgenic fetus. Other experiments suggest to us that cells of the mid-region, as described below in the

Examples, might be the most effective. Alternatively, the cells of the base of the allantois seem to be more pluripotent and these cells might be selected for other applications.

5 Additionally, the Examples below suggest that the location of the cell transplant can determine allantoic cell development. Other applications might require that the allantois cells be transplanted specifically to a particular location to achieve redirected allantoic
10 development, according to the new host site. For example, allantoic cells from the appropriate allantoic region may be placed into the liver where the liver redirects allantoic cells to differentiate into the endothelium characteristic of the vasculature of liver
15 cells.

 In a particularly advantageous form of the present invention, one obtains allantois cells compatible with the fetus and transplants the allantois cells to the fetus wherein the transplanted cells develop into
20 endothelial cells which line the vasculature of the umbilical cord and release the desired gene product into the bloodstream of the fetus.

 It is useful in the Examples below to work with cells containing a marker gene, such as the LacZ gene.
25 The presence of a marker gene enables one to easily monitor whether the foreign cells have integrated with the native allantois.

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Once appropriate allantois cells have been produced, the cells will be injected into the exocoelomic cavity of the defective embryo or transplanted directly onto a particular region of the fetus. Assimilation of the
5 cells into the native allantois should commence and thus, delivery of factor VIII to the fetus. Preferably, the cells will be transplanted directly into the umbilical cord or into a particular region of the fetus.

For human gene therapy, allantois or fetal umbilical
10 cord cells may be obtained from an aborted or miscarried fetus.

The present invention is also a method of fetal gene therapy comprising identifying a fetus with a genetic defect, and obtaining allantois or umbilical cord cells
15 compatible with the fetus, wherein the cells express a gene product that ameliorates the genetic defect. These cells are also capable of colonizing a fetal organ. One then exposes the fetus to the cells. A chimeric fetal organ capable of supplying the gene product to the fetus
20 is created. By "organ" we mean to include the blood circulatory system. Preferably, the fetal organ is a liver or aorta. Preferably, the method of exposure is microinjection of the cells into the fetal organ.

2. In Vitro System of Vasculogenesis

25 We have discovered, as demonstrated below in Section II, that the allantois vascularizes by vasculogenesis that is not accompanied by erythropoiesis.

Transformation of core allantoic mesoderm into the

endothelial cells of blood vessels initially occurs along a spatial gradient, with the cells more distal to the embryo farther along in their developmental program than those more proximal (K.M. Downs and C. Harmann, supra, 1997; K.M. Downs, et al., supra, 1998). How core allantoic mesoderm differentiates is not known, though results of our studies strongly implicate allantoic mesothelium as a key player in this transformation, because mesothelial cell formation coincides with, or possibly precedes vascularization in core mesoderm.

Moreover, Flk-1, an early marker of endothelial cells, is expressed only in core mesoderm, whereas Flk-1's ligand, vascular endothelial growth factor (VEGF), is expressed in the overlying mesothelium, invoking a paracrine system of differentiation between mesothelium and core mesoderm.

Therefore, in one embodiment, the present invention is a method for evaluating the effect of test compounds, preferably potentially harmful or beneficial substances, on formation of blood vessels during vasculogenesis. This method may comprise direct application of a test compound to cultured allantoic explants, as described in Section II and U.S. Serial No. 08/838,384. In another embodiment, one would evaluate test compounds by DNA uptake and expression of a test compound by the mesenchymal cells of allantoic explants.

Inhibitors of vasculogenesis are known to affect angiogenesis (see M.S. Pepper, et al., Cytokine and

Growth Factor Reviews 8:21-43, 1997). Pepper, et al.

show that transforming Growth Factor beta (TGFbeta) has different functions on vessel formation at different stages in the process and regulates formation of blood vessels (vasculogenesis) by potentiating or inhibiting the activity of positive regulators such as basic Fibroblast Growth Factor (bFGF) and Vascular Endothelial Growth Factor (VEGF) in a concentration-dependent manner. On the other hand, once a vessel has formed, TGFbeta1 promotes maintenance of endothelial cell quiescence and induces vessel maturation (both vasculogenesis and angiogenesis). Therefore, we expect that the test compounds found to modulate vasculogenesis will modulate angiogenesis as well.

15 Relevance of the Mouse Conceptus as a Model System for Development of the Chorioallantoic Placenta

Many eutherian mammals, including humans and rodents, survive and develop within the uterine environment through the formation of a chorio-allantoic placenta. Although fine details may vary, all placentae contain an umbilical circulation that consists of at least one major artery and vein. Without exception, these major blood vessels transport fetal blood to and from the chorionic disc for the exchange of nutrients, metabolic wastes and gases with the mother (H.W. Mossman, Vertebrate Fetal Membranes. MacMillan Press Ltd: Basingstoke, UK, 1987).

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The mouse is an ideal model system for the study of umbilical development for several reasons. First, formation of the placenta occurs on schedule in whole embryo culture of living mouse conceptuses (K.M. Downs and R.L. Gardner, supra, 1995; K.M. Downs, et al., supra, 1998; K.M. Downs, et al., supra, 1999). Second, the allantois, precursor of the umbilical cord, is particularly amenable to manipulation *in vitro* and can be isolated free of contamination from the conceptus (K.M. Downs and R.L. Gardner, supra, 1995; K.M. Downs and C. Harmann, supra, 1997; K.M. Downs, et al., supra, 1998; K.M. Downs, et al., supra, 1999; reviewed in K.M. Downs, supra, 1998). Third, transgenic mouse technology has enabled the identification of genes involved in formation of the placenta, either because its two major components, the allantois and the chorion, do not unite in the mutant mice (G.C. Gurtner, et al., Genes and Dev. 9:1-14, 1995; L. Kwee, et al., Development 121:489-503, 1995; J.T. Yang, et al., Development 121:549-560, 1995) or because vasculogenesis has not occurred in the umbilicus (R.J. Akhurst, et al., Development 108:645-656, 1990; M.C. Dickson, et al., Development 121:1845-1854, 1995; F. Shalaby, et al., Nature 376:62-66, 1995). Thus, the mouse is an ideal system in which to elucidate the genetic control of major developmental processes. There exists no other mammalian model at this time that exhibits all of these significant strengths.

Description of the Technique of *in vitro*
Vasculogenesis, Applications, and Preliminary
Results

We have recently demonstrated that the murine
5 allantois vascularizes via vasculogenesis, an intrinsic
process in which mesoderm is transformed into the
endothelial cell lineage *in situ*, rather than by
angiogenesis, which is the extension of blood vessels
formed elsewhere (K.M. Downs, et al., supra, 1998).
10 Further, unlike all other vasculogenic systems heretofore
described (reviewed in W. Risau and I. Flamme, Ann. Rev.
Cell Dev. Biol. 11:73-91, 1995), allantoic vasculogenesis
is not accompanied by erythropoiesis, i.e., formation of
red blood cells.
15 In the course of our studies, we demonstrated that
when allantoises are removed from headfold-stage
conceptuses (approximately 8.0 days postcoitum) and
cultured under relatively simple conditions in isolation,
they rapidly undergo reproducible and stereotypic
20 vasculogenesis (K.M. Downs, et al., supra, 1998, 1999).
With feeding, the allantoic vasculature is maintained and
remodeled for up to 3 days. The cultured explants
consist of at least three cell lineages, endothelial,
mesothelial, and mesenchymal, all of which are normally
25 found in intact allantoises. Further, correct
topographical relations between at least two of these
lineages, the endothelial and mesothelial cells, are
maintained in the explants. Moreover, cells from
explanted cultured allantoises can be returned to

developmentally-equivalent host allantoises where they
correctly colonize appropriate cell types. Lastly, one
of the explanted cell populations, the mesenchymal cells,
can take up and express exogenous DNA. On the basis of
5 our findings, we propose that the murine allantois will
be a powerful and extremely valuable model system for at
least two novel applications (Method 1 and Method 2),
described below:

10 Method 1. Evaluation of potentially harmful/
 beneficial substances on formation of blood
 vessels during vasculogenesis.

 In one embodiment of the present invention,
allantoic explants will be used to evaluate the effect of
potentially toxic environmental compounds and specific
15 gene products on either abrogation or enhancement of
blood vessel formation.

 Two preferred methods are envisioned to achieve this
goal: (a) direct application of a test compound to the
cultured allantoic explants or (b) DNA uptake and
20 expression of a test compound by the mesenchymal cells of
allantoic explants. We envision that the allantoic
explants will, preferably, be created as described below.

 In general, allantoises are mouth-aspirated into a
hand-pulled glass microcapillary (K.M. Downs and R.L.
25 Gardner, supra; K.M. Downs, et al., supra, 1998) and
either cultured in suspension (K.M. Downs, et al., supra,
1998) or placed individually either into wells of 24-well
tissue-culture plats (Falcon 304), or onto glass cover
slips (12 mm, Fisher) inserted into wells of 24-well

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tissue-culture plates and coated for 30 minutes in
filtered (0.45 μ m, cellulose acetate) poly-D-lysine (1
mg/ml double processed tissue culture water; Sigma) and
rinsed 3-4 times with sterile water.

5 Allantoises are then cultured in 0.5 ml of culture
medium (Dulbecco's modified Eagle's medium (DMEM)
containing either (i) equal parts of immediately-
centrifuged and heat-inactivated rat serum as previously
described (K.M. Downs and R.L. Gardner, supra, 1995), or
10 (ii) varying concentrations of heat-inactivated rat or
fetal calf serum (the latter obtained from Gibco-BRL;
frozen and thawed twice before using). For culture
longer than one day, allantoises are given completely
fresh medium at 24-hour intervals.

15 At the onset of culture, or at varying times
thereafter, known test compounds, such as Vascular
Endothelial Growth Factor (VEGF, R & D Systems,
Minneapolis, MN), are prepared according to the
manufacturer's instructions and added to the cultures in
20 concentrations recommended by the manufacturer (e.g., 1-
20 ng VEGF/ml culture medium) or, in the case of unknown
test compounds, in varying concentrations to determine
the one that either inhibits or enhances vasculogenesis.

The advantage of technique (a) is that a controlled
25 amount of substance can be delivered to the allantois at
specific and different times in vascular development and
its effect on vascularization assessed. Moreover, that
compound can be removed at particular times in order to

assess the long-term affect of the compound on the formation of blood vessels.

For technique (b), one would preferentially begin by culturing individually-plated headfold-stage allantoises
5 for 12 or 18 hours in 24-well dishes before transfection, at which time 0.5 ml of heat- and gas-equilibrated DMEM containing 5% fetal calf serum (Gibco-BRL) replaces the culture medium. Allantoises are transfected using a standard CaHPO_4 method (F.L. Graham and A.J. van der Eb,
10 Virology 54:536-539, 1973) for 6 hours in 5.0% CO_2 at 37°C by addition of 50 μ of precipitate containing, for example, 1 μg of plasmid containing the test gene of interest whose expression is driven by the immediate early promoter of human cytomegalovirus (Clontech, Palo
15 Alto, CA). Controls are explants cultured in either DMEM containing 50% rat serum or 5% FCS for the 6 hour transfection period. Following incubation, allantoises are washed once with warm PBS and returned to incubate for varying periods at 37°C in 6.2% CO_2 in DMEM containing
20 50% rat serum to assess the affect of the test compound on vascularization. Expression of the gene of interest is monitored by immunohistochemistry.

The advantage of technique (b) is that, for compounds with relatively short half-lives, the
25 mesenchymal cells of allantoic explants can take up the gene whose product is of interest and express that product continuously in the culture to assess its affect

on growth, development and/or maintenance of the vasculature.

Vascular Endothelial Growth Factor (VEGF) is expressed in the allantoic mesothelium (D.J. Dumont, et al., Dev. Dyn. 203:80-92, 1995) before spreading into the core (K.M. Downs, unpublished data). We have demonstrated that culture of allantoic explants in high rat serum (20-50% rat serum) is optimal for the formation of blood vessels (K.M. Downs, et al., supra, 1999, in revision). Culture of explants in low serum (fetal calf serum, FCS, 5-10%) favors formation of angioblasts, as revealed by expression of Flk-1 and Flt-1, early markers of angioblasts, but not their conversion into nascent blood vessels (K.M. Downs, et al., 1999, in revision). Moreover, despite feeding, allantoises cultured in 5% FCS are typically devoid of vascular channels by 48 hours. By 72 hours, explants cultured in and fed 5% FCS at 24 hour intervals consist predominantly of mesenchymal cells. Increasing the concentration of FCS to 10-20% FCS results in partial maintenance of vascular channels for up to 72 hours, though significant breakdown of the channels is observed in about 87.5% of explants. Thus, a high concentration of some factor(s) must be required for both formation and maintenance of endothelial cells in allantoic explants. To test that possibility, recombinant VEGF (1-10 ng/ml culture medium) was added to explants at the start of culture in 5% FCS. Feeding at 24 hour intervals in the presence of Vascular Endothelial

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Growth Factor (2-10 ng/ml) resulted in formation of many vascular channels containing Flk-1 and Flt-1, and cell survival (78% cell retention compared with 36% in untreated explants) whereas untreated explants or those
5 treated with 1 ng/ml of VEGF were devoid of such channels.

Together these findings suggest that varying the culture conditions of allantoic explants through serum starvation or enrichment varies the state of the
10 endothelial cells, with low serum favoring formation of non-epithelialized angioblasts, and high serum favoring formation of endothelial channels. At least one of the key growth factors required in formation and maintenance of vascularity appears to be VEGF.

15 Method 2. Amelioration/elimination of developmental defects through delivery of blood-borne therapeutic factors into the umbilical circulation by formation of chimeric allantoises.

20 In another embodiment, the present invention is the delivery of factors into the umbilical circulation by formation of a chimeric allantois. Preferably, this delivery results in a therapeutic effect or the amelioration or elimination of developmental defects.

25 I have recently proposed that cultured allantoic cells may be a valuable source of genetically-manipulable cells that could be re-introduced into developing allantoises where they would express a therapeutic gene and deliver it to the fetal bloodstream for the
30 amelioration/cure of certain developmental defects (K.M.

Downs, supra, 1998). We have recently demonstrated that cells of cultured explanted allantoises can be returned to the nascent umbilical cord where they integrate into all three allantoic cell lineages, endothelium,

5 mesothelium and mesenchyme. At least 34% of injected cells colonize the host allantois. In addition, 5.5-6.0% of mesenchymal cells in allantoic explants are able to take up and express exogenously introduced DNA. Mesenchymal cells are thought to be relatively
10 undifferentiated. Then, following transfection of mesenchymal cells with therapeutic genes expressed from appropriate endothelial cell promoters, we propose that donor mesenchymal cells will be introduced into the developing allantois of a developmentally-compromised
15 fetus and, once integrated into the vasculature, they will express the therapeutic compound and deliver it directly into the bloodstream of the affected fetus.

The following is a preferred method of the present invention: Individually-plated headfold-stage
20 allantoises are cultured for 12-18 hours in 24-well dishes before transfection at which time, 0.5 ml of heat- and gas-equilibrated DMEM containing 5% fetal calf serum (Gibco-BRL) replaces the culture medium. Allantoises are transfected using a standard CaHPO₄ method (F.L. Graham
25 and A.J. van der Eb, supra, 1973) for 6 hours in 5.0% CO₂ at 37°C by addition of 50 µl of precipitate containing the plasmid of interest driven either by the immediate early promoter of human cytomegalovirus or the TIE1

endothelial cell specific promoter (Korhonen, et al.,
Blood 86:1828-1835, 1995; T.M. Schlaeger, et al.,
Development 11:1089-1098, 1995). Following incubation,
allantoises are washed once with warm PBS and returned to
5 incubate for 20-24 hours at 37°C in 6.2% CO₂ in DMEM
containing 50% rat serum. Antibodies to the gene product
of interest are applied to some of the cultures to ensure
that transfection has taken place and that the gene of
interest is being expressed.

10 Transfection is the process of macromolecule
transfer to cells by physical or chemical means. One may
decide to use various methods of transfection in the
method of the present invention. For nearly twenty
years, physical, chemical and viral-based methods have
15 been widely available for introducing DNA into mammalian
cells in culture. Physical methods may employ
high-voltage electric pulses to create pores in membranes
("electroporation"; E. Neumann, et al., EMBO J. 1:841-
845, 1982) or a gun to "shoot" genes into individual
20 cells. Most commonly, chemical methods such as calcium
phosphate or DEAE-dextran (or its analogues) are used as
carrier to deliver DNA into cells (F.L. Graham and A.J.
van der Eb, supra, 1973); alternatively, cationic
liposomes containing DNA within them fuse with cell
25 membranes to deliver DNA (J.P. Behr, et al., Proc. Natl.
Acad. Sci. USA 86:6982, 1989; J.P. Loeffler, et al., J.
Neurochem. 54:1812-1815, 1990; F. Barthel, et al., DNA
Cell Biol. 12:553, 1993; J.S. Remy, et al., Bioconjugate

Chem. 5:647-654, 1994). Virus-mediated transfer involves host-specific viruses that either replicate and express DNA in the cytoplasm as episomes (e.g., adenoviruses) or that integrate into the host's genome (e.g., retroviruses).

In the present invention, transfection by calcium phosphate and cationic liposomes (lipofection) will be the two methods of choice used to determine uptake and efficiency of DNA transfer to cultured allantoic cells. These are the most reliable, cost-efficient, and safe means by which to introduce DNA into mammalian cells. However, virus-based transfer is also suitable.

Calcium phosphate precipitation (F.L. Graham and A.J. van der Eb, supra, 1973; M. Wigler, et al., Cell 16:777-785, 1977) and lipofection (J.P. Behr, et al., supra, 1989; J.P. Loeffler, et al., supra, 1990; F. Barthel, et al., supra, 1993; J.S. Remy, et al., supra, 1994; Delaplace, 1991) have both been described and reliable and practical kits for both of these methods are available (e.g., ProfectionR Mammalian Transfection Systems and TfxTM products, Promega, Fitchburg, WI). Our overall strategy for transfection will involve a timecourse, i.e., application of the reporter gene between the time of removal of the allantois from the conceptus and up to 24 hours in culture.

Calcium phosphate-mediated transfection involves mixing DNA directly with CaCl₂ and a phosphate buffer to form a precipitate that is added to the cultured cells.

This method achieves both transient and stable expression of DNA, the latter following integration of the transfected DNA into the host cell genome (M. Wigler, et al., Cell 16:777-785, 1979; M. Botchan, et al., Cell 20:143-152, 1980; S. Kato, et al., Mol. Cell. Biol. 6:1787-1795, 1986) or by autonomous replication in mini-chromosomal structures (D.H. Hamer, et al., Cell 17:725-735, 1979; D. DiMaio, et al., Proc. Natl. Acad. Sci. USA 79:4030-4034, 1982; R. Reeves, et al., Nucl. Acids Res. 13:3599-3615, 1985). As described above, allantoises will be removed and plated in individual wells of 24-well tissue culture dishes. One or more allantoises will be plated per well. Because CaP-mediated transfection requires that cells be 30-60% confluent, allantoises will be cultured for 12 hours, which is ample time for them to flatten out and spread somewhat on the bottom of the dish (R. Temkin, et al., Development, 1998). Prior to transfection, the culture medium will be changed to medium containing 5% fetal calf serum, and the CaP/GFP complex added to the wells. The cells will be returned to the incubator and exposed to the precipitate for 6 hours, after which they will be washed with phosphate buffered saline (PBS, Sigma) and exposed to fresh media. They will then be re-fed every 24 hours up to the time of analysis, which will take place 36-54 hours after transfection (54-72 hours total time in culture). To increase the efficiency of transformation, some of the available "shock" methods,

such as application of DMSO, will be applied to the cell cultures 14-16 hours after transfection and immediately removed and replaced with fresh medium.

Delivery of DNA into the nucleus of allantoic cells
5 via lipofection involves close association of the
cationic liposome-DNA complex with the cell membrane,
followed by internalization of the complex into the cell,
perhaps by fusion with the cell membrane and endocytosis
(X. Gao and L. Huang, Gene Ther. 2:710-722, 1995; P. Hug
10 and R.G. Sleight, Biochim. Biophys. Acta 1097:1-17,
1991). This method results in both transient and stable
transfection. GFP-plasmid (as a control) and a plasmid
of interest will be combined with lipofection reagents
(e.g., TfxTM reagent, Promega, Fitchburg, WI) according
15 to the manufacturer's instructions. The complexes will
then be added to allantoic cell cultures at different
timepoints following removal and plating of the
allantois. We will do this in both the presence (10%
FCS) and absence of serum. Lipofection in the absence of
20 serum is more efficient, but this may of course be, cell
line dependent. The advantage of this method of delivery
is that two hours, instead of six, is all the time needed
to expose cells to the exogenous DNA.

It is possible that chemical methods will not
25 achieve the highest frequency of transfection and
expression in whole allantoises. In that case,
alternative strategies may be used, which employ
viral-mediated DNA transfer. Infection with viruses

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would be preferred over microinjection of DNA into the nucleus because the latter method is labor intensive and can target only a small number of cells at a time. The general methodology would be similar to that described

5 above for transfection. Allantoises would be plated and soon thereafter infected according to standard protocols with a high titer virus genetically engineered with a reporter gene, for example, GFP whose genetic size is suitable for most viral vectors. Scoring would be as

10 described above, and infected cultures trypsinized and introduced into living embryos to assay for appropriate integration into the vasculature and gene expression.

Most viral vectors have common limitations. Among these are the size of the foreign gene that they may

15 accommodate (maximal insert sizes for SV40 and retroviruses are 2.5 kb and 6 kb, respectively), and/or the fact that they may be subjected to rearrangement upon propagation of the viral stock, a serious consideration in the case of adenoviruses (H. Lochmuller, et al., Hum. Gene Ther. 5:1485-1491, 1994) and requiring therefore

20 constant monitoring of viral stocks. Another limitation is the cytopathic effect of some viruses, particularly adenoviruses, on the host cell, which limits expression to a relatively short period of time. Finally, the

25 variability in gene expression depends upon many parameters which are not completely clear. Among these are proper translation, processing, and modification of the resulting protein.

The viral vectors of choice will be adenovirus (Karpati, et al., 1996; Yeh and Perricaudet, 1997) and helper-virus-free retrovirus vectors (K. Shimotohno and H.M. Temin, Cell 26:66-77, 1981).

5 Significance and Summary

Use of the murine allantois as a model *in vitro* system of vasculogenesis will have enormous impact on the study of formation of blood vessels for several reasons. First, abnormalities during the development of two major
10 vascular systems, the heart and circulation, are the leading cause of birth defects (March of Dimes Web Page, 1999). A valuable approach for elucidating the cellular and molecular mechanisms of endothelial cell formation would be the discovery of practical and reproducible *in*
15 *vitro* systems of vasculogenesis. Embryonic stem cells from mice (W. Risau, et al., Development 102:471-478, 1988; R. Wang, et al., Development 114:303-316, 1992; R.L. Gendron, et al., Dev. Biol. 177:332-346, 1996; D. Vittet, et al., Blood 88:3424-3431, 1996) and avian
20 epiblast (I. Flamme and W. Risau, supra, 1992; I. Flamme, et al., Anat. Rec. 237:49-57, 1993; K. Krah, et al., Dev. Biol. 164:123-132, 1994) have been used to this end. A major drawback to these is that differentiation of embryonic stem cells into the endothelial cell lineage is
25 always accompanied by erythropoiesis. The presence of blood cells makes it difficult to determine which factors are essential for vasculogenesis alone. In addition, the types of cells present and their topographical

relationships to each other vary from culture to culture, differentiation occurs over relatively long time periods, and the frequency of vasculogenesis/erythropoiesis is typically much lower than 100 percent. Pure cultures of

5 endothelial cells have been isolated but, because most of them have been derived from adult organs (e.g., J.D. Rone and A.L. Goodman, Proc. Soc. Exp. Biol. Med. 184:495-503, 1987; L.C. Masek and J.W. Sweetenham, Brit. J. Haem. 88:855-865, 1994; K. Uchida, et al., Am. J. Physiol. 266:F81-88, 1994; G. Haraldsen, et al., Gut 37:225-234, 1995; T. Sakamoto, et al., Curr. Eye Res. 14:621-627, 1995; L.K. Christenson and R.L. Stouffer, Biol. Reprod. 55:1397-1404, 1996; Q. Yan, et al., Invest. Ophthal. Vis. Sci. 37:2185-2194, 1996), they offer little in the way of

10 recapitulating the early steps of vasculogenesis.

Second, although vasculogenesis and angiogenesis are typically described as distinct developmental process, it is not known in what way, if any, these processes involve different factors. Angiogenesis, which is the extension

15 of blood vessels from elsewhere, is critical for the growth and metastatic spread of tumors. In the absence of a blood supply, tumor size remains fixed; without access to the vasculature, metastatic tumor cells are denied access to travel about the body. Vascularization

20 of tumors involves the microvasculature, composed of endothelial cells. Comparison of the effect of particular proteins on the formation of endothelial cells via vasculogenesis in the allantoic explants with current

models of endothelial cell formation via angiogenesis may lead to the identification of tumor-specific angiogenic proteins (reviewed in R. Auerbach, Int. J. Radiat. Biol. 60:1-10, 1991).

5 Lastly, the ability of allantoic cells to be genetically-manipulated and to colonize the developing allantois may prove therapeutically valuable for *in utero* gene therapy in cases where a blood-borne circulating factor might ameliorate or cure certain fetal defects.

10

EXAMPLES

I. Method of Delivering Therapeutic Molecules to a Fetus

In General

15 In the following examples, we examine the fate and developmental potency of cells within the murine allantois during gastrulation.

20 LacZ-expressing headfold-stage (approximately 8 days postcoitum, dpc) allantoises were subdivided into three prosimodistal regions and transplanted in three sites in synchronous non-transgenic host embryos at sites where the fate of cells was largely known: (1) the base of the allantois (called "orthotopic" and "approximate orthotopic" grafts, R.S.P. Beddington, 1981, 1982), (2) the prospective lateral plate mesoderms of the fetus
25 (called "heterotopic" grafts (R.S.P. Beddington, supra, 1982), and (3) the prospective paraxial mesoderm of the fetus ("heterotopic grafts). The rationale behind partitioning the allantois was that two of the regions

appear to have different functions: the tip is involved in chorioallantoic fusion (K.M. Downs and R.L. Gardner, supra, 1995) and the base may induce or protect presumptive primordial germ cells (PGCs) from differentiation (A. McLaren, Bibthca Anat. 24:59-66, 1989; A. McLaren, Andrologia 24:243-247, 1992). After 23 hours of whole embryo culture (K.M. Downs and R.L. Gardner, supra, 1995) control and operated conceptuses were stained with benzidine and X-gal, prepared for histology, and examined.

When placed into Site (3), none of the allantoic regions contributed to paraxial mesoderm, but all of them colonized the endothelium of the dorsal aorta and surrounding periaortic mesenchyme when placed there and into Site (2). Thus, these results reveal that angioblasts are present in the allantois by the headfold stage.

Further, striking differences were observed in the state of differentiation of donor allantoic cells dependent upon their original location along the allantoic proximodistal axis. Cells in the base of the allantois were relatively pluripotent, contributing additionally to endoderm of the future coelom and surface ectoderm, whereas cells for the allantoic mid-region exhibited the greatest vasculogenic potential. Donor allantoic tip cells returned most often to the distal region of the allantois when placed into Site (1), the base of the allantois.

Our data suggest a model of angioblast formation which states that the genetic changes required for transformation of pluripotent mesodermal cells into differentiated angioblasts commence with increasing distance from the subjacent posterior primitive streak.

Lastly, of potential significance was our finding that allantoic cells, in particular basal ones, do not return to the fetus. Thus, the allantois is a self-contained vasculogenic system.

10 Use of the Pre-umbilical Cord and Derived Cell Lines in Gene Therapy of the Fetus.

One objective of the present invention is to use the umbilical cord for fetal gene therapy *in utero* of congenital defects in blood-borne factors. The umbilical blood vessels provide a direct gateway to the fetal circulation and a very attractive and potentially invaluable system for continuous delivery of therapeutic factors to the fetus.

Although the ultimate goal of this invention is to treat human fetuses with congenital defects *in utero*, for ethical and practical reasons, the soundness of the approach will first be demonstrated in an animal model. The mouse was chosen for this work because of the similarity of its umbilical cord to the human's, because the murine embryo can be manipulated both *ex utero* (K.M. Downs and R.L. Gardner, supra, 1995) and *in utero* (V.E. Papaioannou, *In utero* manipulations, In Postimplantation Mammalian Embryos, A Practical Approach, eds. A.J. Copp

and D. L. Cockroft, Oxford: Oxford University Press, 1990), and because the ease of manipulation of the mouse genome has provided excellent models of human genetic disease.

5 These results suggested that with little manipulation, the native umbilical cord might be an ideal vehicle for delivery of therapeutic substances to developmentally-compromised fetuses.

10 Delivery of Therapeutic Substances to the Fetus:
 The Test Model.

 The success of our microsurgery in living conceptuses described above has led to the hypothesis that the umbilical cord can be manipulated for therapeutic purposes (see also K.M. Downs and R.L. Gardner, supra, 1995). These results, and others which have demonstrated gene expression from endothelial cell-specific promoters (J. Korhonen, et al., supra, 1995; T.M. Schlaeger, et al., supra, 1995) suggest to me that the umbilical cord has the potential to express therapeutic factors in the endothelial cells that comprise its blood vessels and deliver them to the fetal circulation.

 We propose to first demonstrate the present invention in transgenic mice with Haemophilia A. Our ultimate goal is to eliminate antibody production against Factor VIII in human patients with Haemophilia A. Haemophilia is the most common of the inherited bleeding disorders. It affects 1 in 5-10,000 males in all

populations and is caused by a defect in clotting Factor VIII. Factor VIII has been cloned and is expressed in many cell types, including mouse fibroblast cells (J.N. Lozier and K.M. Brinkhous, JAMA 271:47-51, 1994). There
5 is no precise regulation of the clotting proteins, and no tissue-specific requirement (D.I. Israel and R.J. Kaufman, Blood 75:1074-1080, 1990). Because many patients with Haemophilia A will develop inhibitors against recombinant Factor VIII (L. Bi, et al., Nature
10 Genetics 10:119-121, 1995), a possible means for preventing formation of these antibodies would be to begin therapy *in utero*, as the immune system is developing. This would ensure that Factor VIII is not recognized as a foreign protein when given after birth.
15 At present, the only methods available for introducing the fetus to therapeutic factors are the mother's circulation or intermittent injection into the umbilical cord. However, Factor VIII will not cross the placental barrier (V. Mahnovski and Z. Pavlova, In Diseases of the
20 Fetus and Newborn: Pathology, Radiology, and Genetics, eds. G.B. Reed, A.E. Clareaux, and A.D. Bain). St. Louis: The C.V. Mosby Company, pp. 417-440, 1989), and injection into the umbilical cord is impractical (half-life of Factor VIII protein is 10 hours, G.G. Brownlee, Prospects
25 for gene therapy of Haemophilia A and B, In Gene Therapy (eds. A.M. Lever and P.G. Goodfellow), New York: Churchill Livingstone, 1995) and costly. All of these

characteristics make Haemophilia A an excellent candidate disease for the gene therapy of the present invention.

i. Overall Experimental Design.

5 The success of fetal gene therapy via the umbilical
cord rests upon obtaining allantois cell lines whose
properties are similar to intact allantois cells and
which can be manipulated to express therapeutic genes of
interest. For this, allantoises from transgenic mice
that express β -galactosidase (LacZ) in all their cells
10 (G. Friedrich and P. Soriano, supra, 1991) will be
removed and placed in culture. Explants and their LacZ-
expressing cells will be selected and infected with a
retrovirus containing human Factor VIII (D.I. Israel and
R.J. Kaufman, supra, 1990). These LacZ/Factor VIII-
15 expressing cells will then be introduced into the
exocoelomic cavity of mice defective in Factor VIII (L.
Bi, et al., supra, 1995). The fetuses will be monitored
through gestation for production of Factor VIII in their
serum and antibodies against Factor VIII (L. Bi, et al.,
20 supra, 1995). A schematic diagram of the overall
experimental design is shown in Fig. 2.

ii. Construction of Factor VIII under
Endothelial Cell-Specific Promoters in a
Retroviral Vector.

25 Factor VIII is produced in the liver, though its
exact site of production is still controversial (J.E.
Sadler and E.W. Davie, In The Molecular Basis of Blood
Diseases, 2nd Edition, eds. G. Stamatoyannopoulos, A.W.

(J. Korhonen, et al. supra, 1995) or *tie-2* (T.M. Schlaeger, et al., supra, 1995) in a retroviral vector defective in replication (R.G. Vile and S.J. Russell, In Gene Therapy, eds. A.M. Lever and P.G. Goodfellow, New York: Churchill Livingstone, 1995). These retroviruses will also carry a selection marker, possibly neomycin, thymidine kinase, metallothionein or adenosine deaminase (Ada) in order to select against uninfected cells. Infection of LacZ-allantois cells with retrovirus containing Factor VIII will be either in whole allantois explants or in cell lines derived from the explants (see next section). Infected allantois cells will be subcloned and duplicated in order to test for (i) LacZ expression, (ii) karyotype, and (iii) expression of Factor VIII protein by the COATEST (D.I. Israel and R.J. Kaufman, supra, 1990). Different cell lines will be compared for the highest production of Factor VIII.

iii. Creation of LacZ/Factor VIII-Expressing Cell Lines.

LacZ-expressing and LacZ/Factor VIII-expressing allantois cell lines will be created. LacZ cells express β -galactosidase activity that can be visualized with the application of X-gal, turning the cells blue. Thus for the pilot experiments proposed here, we will be able to monitor the behavior of our cell lines when introduced into living embryos.

Whole allantoises from LacZ-expressing conceptuses will be removed with glass capillary pipettes (K.M. Downs

and R.L. Gardner, supra, 1995; K.M. Downs, et al.,
Nature, submitted, 1996) and placed into tissue culture
dishes. At first, media containing 15% fetal calf serum
will be used, but upon passage, that amount will be
5 reduced to 10% (R.I. Freshney, Culture of Animal Cells.
A Manual of Basic Technique, New York: Alan R. Liss,
Inc., 1983). The following parameters will be varied in
order to obtain primary cultures: disaggregation/no
disaggregation of the whole allantois before explanting
10 to a tissue culture dish, culture media, dish matrices
and stage of allantois removal from the conceptus.
Ideally, outgrowths from younger allantoises would be
most desirable, as young allantoises are more likely to
contain cells that are stem-cell-like. Also, it would be
15 most desirable to obtain a cell line with a normal
karyotype (i.e., 40 chromosomes) because such cells
should behave normally when eventually re-introduced into
the living conceptus.

Once a reasonable number of cells have grown out,
20 they will be passaged and subcloned. Duplicate subclones
will be tested for LacZ expression and normal karyotype.
Cells expressing LacZ and which have maintained a normal
karyotype will be introduced into the exocoelom of a
living embryo and assessed for integration into the
25 umbilical cord after appropriate growth of the "operated"
conceptus in culture.

If we are unable to obtain karyotypically-normal
primary cultures of LacZ/Factor VIII-expressing allantois

cells, we will attempt to immortalize allantois cells by introducing explants to replication-defective retroviruses which express SV40 large T antigen, known to immortalize many different cell types *in vitro* (M. Noble, et al., Transgenic Research 4:215-225, 1995) and a selectable marker different from the one used with Factor VIII. Different cell lines will then be introduced into host conceptuses to test for normal behavior and manipulated with Factor VIII as described below.

10 Infection of cells with a retrovirus carrying Factor VIII will take place either when explants are fairly large (approximately one week) or when a LacZ-allantois cell line has been obtained. Ideally, they will be infected with a defective retrovirus containing Factor
15 VIII and a selectable marker, either neomycin, thymidine kinase or adenosine deaminase (see below). Twenty-four hours after infection, explants will be disaggregated and passaged until cells reach confluence.

20 iv. Formation of Chimeric Umbilical Cords in utero and Delivery of Factor VIII to Hemophilic Fetuses.

The second set of experiments will be carried out *in utero* (V.E. Papaioannou, supra, 1990) in transgenic mice deficient in Factor VIII (L. Bi, et al., supra, 1995).
25 Haemophilia is an X-linked disease, so only about half the males of the litters from the mating ($X+X^H \times X+Y$) will be defective in Factor VIII. LacZ-/Factor VIII-expressing allantois cell will be introduced into the exocoelom in all implantation sites and become

incorporated into the native allantois. These chimeric allantoises will develop into the mature umbilical cord, in which a large portion of donor endothelial cells will express Factor VIII. Because the endothelial cells of blood vessels are very long-lived, Factor VIII will be delivered continuously to the fetal bloodstream throughout gestation.

Because the umbilical cord will be shed at birth and will be the only source of Factor VIII in this experimental design, fetuses must be examined for the presence of Factor VIII before birth. Because we will have no way of knowing which fetuses are the hemizygous affected males, all littermates will be examined. The Factor VIII gene that is eventually introduced into the allantois cells will be engineered in such a way that hemizygous males carrying the mutation in Factor VIII can be distinguished from normal and heterozygous littermates who will not be affected by mutated Factor VIII. This strategy has previously been described (L. Bi, et al., supra, 1995).

All fetuses from every operated litter will be removed from their mother at approximately 18 days postcoitum and tested for the following: (i) Levels of Factor VIII in fetal plasma by the COATEST, (ii) Presence of the defective gene in genomic DNA, and (iii) antibodies against Factor VIII.

At birth, the recombinant allantois cells will be shed with the placenta. Continued therapy with Factor

VIII after birth should permit Factor VIII to be recognized as "self" by the adult immune system.

v. Transplantation of Allantoises in the Fetal Liver.

5 For our therapy to reach its full potential, it would be most desirable if allantois/umbilical cells had the potential to colonize fetal/adult organs. In that way, Factor VIII therapy could be continued after birth.

10 Liver cells are the site of Factor VIII synthesis (G.G. Brownlee, supra, 1995; J.E. Sadler and E.W. Davie, supra, 1994). Thus, in initial experiments, LacZ-expressing allantoises will be introduced into fetal and adult livers, which will then be cultured as whole organ explants (I. Parsa and L. Flancbaum, Dev. Biol. 46:120-

15 131, 1975; P.P.L. Tam, In Postimplantation Mammalian Embryos. A Practical Approach, Oxford University Press, Oxford, 1990). After suitable culture, explants will be examined for LacZ-expressing cells. If they are found, then genetically-engineered allantois cells expressing

20 LacZ/Factor VIII will be introduced into the fetal liver *in utero* (V.E. Papaioannou, supra, 1990). *In utero*, the liver is clearly identifiable as a bright red tissue in the trunk of the fetus. If the allantois cells survive transplantation and express genetically-engineered Factor

25 VIII as mRNA distinguishable from native Factor VIII, then trials will take place in which LacZ/Factor VIII cells will be introduced *in utero* into litters from matings between heterozygous defective females and normal

males ($X+X^H \times X+Y$). Newborn pups will be tested for rescue by allantois/umbilical cord cells as previously described (Li, et al., supra, 1995), and for genotype, liver chimerism, and expression of genetically-engineered

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i. Mouse Strains

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were non-transgenic F2's of hybrid F1 (C57BL/6 x CBA) matings.

Mice were kept in 12-hour light-reversed conditions (dark period: 13.00--1.00 hours). Pregnant females were sacrificed by cervical dislocation without prior anesthesia, and the uterine horns dissected into PBS.

ii. Removal and Dissection of Conceptuses

Host conceptuses were dissected at 10.00 on the day of the experiment (approximately 7.75 days post coitum, dpc). In most experiments, only neural plate/late allantoic bud and very early headfold-stage conceptuses were selected as hosts (Fig. 1A; K.M. Downs and T. Davies, supra, 1993; K.M. Downs and R.L. Gardner, supra, 1995). This was to ensure that by the time transplantation was carried out several hours later, most embryos would be at the headfold stage. Conceptuses were paired, and the ectoplacental cone of one member of each pair was trimmed with scissor-motion of two 28 g syringe needles. Trimming the ectoplacental cone enabled the identification of each conceptus after transplantation and culture (K.A. Lawson et al., supra, 1991). Pairs of host conceptuses were then placed in culture medium and maintained in the incubator (K.M. Downs and R.L. Gardner, supra, 1995) until transplantation.

iii. Culture of Allantoises in Isolation

Allantoises of lacZ/+ hemizygous genotype were removed through two vitelline sites (see Fig. 3): (1)

directly through visible yolk sac blood islands, or (2)
through non-blood island tissue slightly distal to the
level of the site of insertion of the allantois with the
amnion. Individual allantoises were then placed in 0.5
5 ml of culture medium (K.M. Downs and R.L. Gardner, supra,
1995) and cultured in roller culture for either 24 or
40-43 hours. After culture, each allantois was stained
in benzidine for 30 minutes to detect
hemoglobin-expressing cells (see Histochemical Analysis,
10 below).

iv. Transplantation

Headfold-stage donor allantoises were removed by
capillary aspiration by piercing the yolk sac just distal
to the site of insertion of the allantois with the amnion
15 (Fig. 3). Fig. 3 is a schematic diagram of the allantois
and delineation of the base at the level of insertion of
the amnion. The delineation of the base of the allantois
with the subjacent primitive streak was determined as the
site of insertion of the amnion (arrow). The solid
20 diagonal line indicates the basalmost portion of the
allantois taken for this study and its point of
separation from the primitive streak (PS) below. The
allantois was subdivided into three regions for
transplantation (dotted lines): the base, the mid-region
25 and the tip. The numbers 1 and 2 indicate yolk sac sites
through which the allantois was removed. Site 1 is
typically free of blood islands observable at the level
of the light microscope whereas Site 2 contains blood

islands. Site 1 was the principal site of yolk sac puncture and removal of the allantois for all of the transplantation experiments described. The bold line surrounding the allantois and which is continuous with the yolk sac and amnion represents the mesothelial lining of the exocoelom. The hatched triangle immediately below the base of the allantois may contain PGC precursors (see Discussion). Abbreviations: AM, amnion; PS, posterior primitive streak; YS, yolk sac.

10 The basalmost portion of the allantois was taken as the site of insertion of the amnion (W. Ozdzenski, Zoologica Poloniae 17:367-381, 1967). Allantoises in which basal cells were not sufficiently removed from attachment to the posterior streak after allantoic aspiration were discarded. Whole allantoises which exhibited distinct basal and distal regions were then rinsed in PBS, and placed individually in trypsin/pancreatin for 5 minutes on ice (B. Hogan, et al., Manipulating the Mouse Embryo. A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Cold Spring, New York, 1994), after which the tip, middle third, or base was excised using glass needles (R.S.P. Beddington, "Isolation, culture and manipulation of post-implantation mouse embryos," In Mammalian Development: A Practical Approach, (ed. M. Monk), Oxford:IRL Press, 1987; Fig. 5). Donor tissue from the embryonic regions included both epiblast and primitive streak but not visceral endoderm.

Transplantations were carried out either as a series of orthotopic grafts only, or as a series of heterotopic grafts which also included a second set of control orthotopic grafts to the base of the allantois. The following host sites were targeted: (1) the base of the allantois, (2) the mid-primitive streak at the level of prospective lateral plate mesoderm, and (3) the more anterior primitive streak at the level of prospective paraxial mesoderm (Fig. 4). Transplantations of the mid- and distal allantoic regions to the base of the allantois were not strictly orthotopic, and therefore they were called "approximate orthotopic" grafts. The point of grafting into the allantois was to control for contribution to the allantois in all experiments where allantoic tissue was placed into the fetus.

Every experiment included unoperated conceptuses and several unoperated transgenic conceptuses in order to compare the extent of development between donor and host embryos (see Morphology of Cultured Conceptuses, below), and to ensure appropriate benzidine and X-gal staining (see Histochemical Analysis). At the time of transplantation, donor tissue was placed in a drop of dissection medium on a glass slide from which the single chamber was removed, leaving behind a silicon gasket (Nunc). The tissue was then cut further into clumps of approximately 10-30 cells; every effort was made to inject all fragments from the desired region; this was successful most of the time. Pairs of recipient

conceptuses to be injected were placed in a second drop of dissection medium. Transplantations were carried out in a Nikon Diaphot inverted microscope with bright-field and DIC optics.

- 5 Two Leitz micromanipulation arms were used to manipulate holding and microinjection pipettes. Holding pipettes (O.D. 1.00 mm, I. D. 0.75 mm) were hand-pulled and heat-polished (R.S.P. Beddington, supra, 1987) on a deFonbrune microforge to an inner diameter of 0.05 mm.
- 10 Microinjection pipettes were pulled on an electrode puller (Sutter Instruments, Novato, CA), broken to an I.D. of 0.03 mm on the microforge, and heat-polished. Holding and microinjection pipettes were mounted on microelectrode holders (World Precision Instruments)
- 15 containing either 0.1 M KCl and silicon oil (holding pipettes) or dissection medium and silicon oil (microinjection pipettes). The electrode holders were connected to Hamilton syringes of 500 μ l (holding pipettes) or 100 μ l (microinjection pipettes).
- 20 All experimental embryos were photographed at the time of transplantation using a video monitor attached to a tv screen (Sony) and printer loaded with black-and-white heat-sensitive paper (Sony) in order to verify retrospectively initial embryonic stage and the
- 25 site of injection.

v. Morphology of Cultured Conceptuses

After culture, each conceptus was scored for the following morphological features (N.A. Brown, "Routine

assessment of morphology and growth: scoring systems and measurements of size," In Postimplantation Mammalian Embryos: a Practical Approach, eds. A.J. Copp and D.L. Cockroft, Oxford:IRL Press, 1990): chorioallantoic fusion, yolk sac circulation, heart beat, turning, extent of development of the gut, brain, heart, and numbers of somite pairs. None of the conceptuses were grossly deficient in any one of these features, but a few exhibited an obvious lack of growth and organization and were judged abnormal (0.65% total cultured conceptuses were not included in the final analysis). The extent of development between unoperated hosts and transgenic conceptuses was also compared in order to verify that the rate of development of donor and host conceptuses was similar. In this analysis, a student T-test revealed no significant differences between development of headfold-stage donor and host conceptuses (scoring based on Brown, supra, 1990; n = 18 (lacZ/+), n = 38 (+/+); p=0.25, equal variances assumed, C. I. = 95.0%).

vi. Histochemical Analysis and Scoring Grafted Cells

After culture, conceptuses and whole allantoises cultured in isolation were immediately stained in benzidine to identify host hemoglobin-synthesizing red blood cells: 20 μ l 30% H₂O₂ (Sigma) was added to 1 ml benzidine stock solution (benzidine stock solution: 50 ml 0.5 M glacial acetic acid plus 100 mg benzidine (Sigma)); 100 μ l of the benzidine/H₂O₂ mixture was added

to 400 μ l phosphate-buffered saline (PBS, Sigma) to
produce a working solution. 100 μ l of the working
solution was added to 300 μ l dissection medium which
contained conceptuses (K.M. Downs and R.L. Gardner,
5 supra, 1995) at room temperature for 15 minutes.
Conceptuses and allantoises were then rinsed twice in PBS
and fixed for 2-2.5 hours at 4°C in 4% paraformaldehyde.
All cells of ROSA26* *lacZ*/+ hemizygous donor headfold-
stage conceptuses were blue after staining in X-gal, both
10 before and after culture (data not shown), with the
exception of an occasional (0.01%) benzidine-positive
donor cell (see below). Fixation periods of 5 hours or
more compromised the ubiquity of X-gal staining in some
cell lineages, particularly yolk sac endoderm, the
15 chorion, and trophoblast giant cells overlying the
ectoplacental cone. Fixed conceptuses were rinsed 3
times in PBS (20 minutes each) and stained in X-gal
(Sigma) at 37°C for at least 15 hours (Sanes, et al.,
supra, 1986). We found that post-fixation in 4%
20 paraformaldehyde after X-gal staining had no visible
effect on retention of the X-gal stain. Conceptuses were
then examined for the presence of transgenic cells before
processing for wax histology by conventional means, which
included vacuum at the paraplast embedding step.
25 Conceptuses were sectioned at 4 μ m thickness
(Reichert-Jung Autocut), and the sections dried at 42°C
for at least 7 hours. Slides were dewaxed by
conventional means, and counterstained in Nuclear Fast

Red (Sigma; MacGregor, et al., Development 121:1487-1496,
1995) for 5 seconds to 1 minute before mounting in
Permount. Nuclei of transgenic donor cells were counted
in every section and locations noted. Operated
5 conceptuses that contained ≥ 5 incorporated cells were
scored as chimeric.

Only red blood cells stained pale yellow to deep
orange if they contained hemoglobin. When co-stained
with X-gal, the overwhelming majority of red blood cells
10 contained significant amounts of X-gal precipitate; some
red blood cells contained significant amounts of X-gal
precipitate; some red blood cells were speckled blue, and
very infrequently ($<0.01\%$), a hemoglobin-positive cell
appeared negative. In the absence of benzidine, all
15 transgenic red blood cells stained blue with X-gal.

Results

Transplantation was used to determine the
developmental potency of the allantois. Donor
allantoises of hemizygous *lacZ*/+ genotype were subdivided
20 into three regions (Fig. 4): base, middle-third, and
tip, and each region was transplanted into three sites in
the conceptus: the base of the allantois, the prospective
lateral plate mesoderm (LPM) and the prospective paraxial
mesoderm (PM) (Fig. 4). These embryonic sites were
25 chosen because they had been previously characterized by
transplantation (P.P.L. Tam and R.S.P. Beddington, supra,
1987), and therefore offered the opportunity to compare
the developmental potential of allantoic extraembryonic

mesoderm in sites where embryonic mesoderm was forming. Most hosts were at the headfold-stage at the time of transplantation, but a small number had reached early somite stages (Figs 5-7). After culture, each chimera
5 was examined for the whereabouts of donor allantoic tissue. Benzidine staining, specific for red blood cells containing hemoglobin, facilitated identification of host endothelial cells.

Fig. 5 is a summary of results of grafts of donor
10 allantoic tissue into the base of host allantoises, headfold stage. The results of transplants of the three donor allantoic regions, tip, middlethird, and base, into the base of the allantois of host conceptuses are shown for every chimera.

15 Fig. 6 is a summary of results of grafts into prospective lateral plate mesoderm, headfold stage. The number and location of donor transgenic cells are shown for every chimera 23 hours after orthotopic (same site) and heterotopic grafts of the three allantoic regions
20 were introduced into the primitive streak at the level of prospective lateral plate mesoderm. The initial and final stages of each chimeric conceptus are shown (HF, headfold; ordinal numbers are pairs of somites).

Fig. 7 is a summary of results of grafts into
25 prospective paraxial mesoderm, headfold stage. This figure summarizes the number and location of donor transgenic cells 23 hours after orthotopic (same site) and heterotopic grafts of the three allantoic regions

were introduced into the primitive streak at the level of prospective paraxial mesoderm. The initial and final stages are as described in Fig. 6.

Abbreviations: MT, middle third of allantois.

5 Orthotopic Control Transplantations

To obtain a profile of the potentially colonizable tissues for each of the three transplantation sites, a set of five orthotopic experiments was carried out, using similar numbers of conceptuses (Table 1A, below). The
10 number of chimeras obtained at these orthotopic sites was within the range of that previously reported (5-12 chimeras; P.P.L. Tam and R.S.P. Beddington, supra, 1987). Embryos receiving grafts from the base of the allantois exhibited the highest rate of chimerism, whereas those
15 receiving grafts at the level of paraxial mesoderm were least efficiently colonized.

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TABLE 1

Expt Set	Site of Injection	Tissue	No. Expts	No. Embs. Injected	No. Neg. Grafts (%)	No. Grafts Unincorp. (%)	No. Chimeras (%)	Mean No. Grafted Cells \pm SEM (Median)	Type of Tissue Colonized
A	O-Base	Base of Allantois	5	20	3 (15.0)	3 (15.0)	14 (70.0)	182.6 \pm 46.2 (115.5)	allantois
	O-LPM	Prospective LPM	5	19	4 (21.1)	5 (26.3)	10 (52.6)	192.5 \pm 68.7 (100.5)	multiple
	O-PM	Prospective PM	5	21	6 (28.6)	8 (38.1)	7 (33.3)	145.9 \pm 61.3 (73)	multiple
B	Base of Allantois	Base of Allantois	4	15	5* (33.3)	3 (20.0)	7 (46.7)	441 \pm 304 (185)	allantois
		Middle Third	4	21	2† (9.5)	3 (14.3)	16 (76.2)	607 \pm 166 (471)	allantois
		Tip of Allantois	4	14	1 (7.1)	1 (7.1)	12 (85.7)	208.4 \pm 82.6 (102)	allantois
	Prospect. LPM	Base of Allantois	4	17	7 (41.2)	6 (35.3)	4 (23.5)	697 \pm 402 (452)	multiple
		Middle Third	4	22	3 (13.6)	7 (31.8)	12 (54.5)	230.8 \pm 60.0 (175)	endothelia, mesoderm
		Tip of Allantois	4	18	2 (11.1)	11 (61.1)	5 (27.8)	199.0 \pm 139.0 (87)	endothelia, mesoderm
	Prospect. PM	Base of Allantois	4	19	7 (36.8)	10 (52.6)	2 (10.5)	68.0 \pm 48.0	endothelia, mesoderm
		Middle Third	4	21	7 (33.3)	8 (38.1)	6 (28.6)	176.7 \pm 75.7 (130)	endothelia, mesoderm
		Tip of Allantois	3	13	1 (7.7)	8 (61.5)	4 (30.8)	85.0 \pm 43.2 (62)	endothelia, mesoderm

5

10

15

Table 1 tabulates the frequency of Experimental
Chimeras and Mean Number of Grafted Donor Descendant
Cells. The bold horizontal lines delineate the types of
transplantations carried out in every experiment. In
5 Experimental Set A, orthotopic (O) transplantations were
carried out to three sites: the base of the allantois,
prospective LPM, and prospective PM. In Experimental Set
B, the allantois was subdivided into three regions, tip,
middle third, and base. Each of these regions was then
10 transplanted to three sites in the same experiment: base
of the allantois, prospective lateral plate mesoderm
(LPM), and prospective paraxial mesoderm (PM).

Abbreviations: Embs, embryos; Expt., experiments; Neg.,
negative; No., number; SEM, standard error of the mean.

15 The * symbol indicates that one graft had only four cells
incorporated into the allantoic endothelium. The †
symbol indicates that one graft had only one cell
incorporated into the allantoic endothelium.

Orthotopic transplantations of the base of the
20 allantois were carried out for the first time. These
revealed the fate of cells in the base of the allantois.
The rate of allantoic chimerism was relatively high
compared with the other two transplantation sites (Table
1A). The higher rate of success with the former was
25 likely due to symmetric apposition and greater distance
between holding and injection pipettes than used with the
other sites. All orthotopically-placed cells from the

base of the allantois contributed exclusively to the allantois. None were found in the fetus (Fig. 5A).

Orthotopic grafts of the region of the primitive streak that gives rise to lateral plate mesoderm resulted in colonization of a wide range of different tissues (Fig. 6). Contribution to non-mesodermal tissues likely reflects the fact that we did not separate the primitive streak from epiblast as did Tam and Beddington (1987), as we did not know whether the allantois would be able to give rise to derivatives of all three primary germ layers rather than to mesoderm alone.

Orthotopic transplantations of the region of the conceptus giving rise to paraxial mesoderm exhibited the lowest rate of chimerism (33.3%, Table 1A) of all orthotopic grafts. However, this number was not significantly different from the overall rate of chimerism for the other two sites. Because transplantations to this site contained a similar number of grafted cells as the other two transplantation sites, the smaller number of chimeras found at the level of prospective paraxial mesoderm is likely explained by destabilization of the injection site due to overlap in the level of the holding and injection pipettes (which, incidentally, we found to be superior than other arrangements of the pipettes at this site). Nonetheless, the number of positive chimeric fetuses was just one less than the number previously analyzed (P.P.L. Tam and R.S.P. Beddington, supra, 1987). Colonization patterns

in chimeric embryos included paraxial mesoderm, as previously described (P.P.L. Tam and R.S.P. Beddington, supra, 1987; Fig. 7).

Developmental Potency of the Allantois

5 Orthotopic and Approximate Orthotopic Transplantation of the Allantois

Given the array of potentially colonizable tissues at each of the three transplantation sites, we then carried out a series of experiments designed to determine
10 the developmental potential of the allantois (Table 2B). Orthotopic and approximate orthotopic transplantations of the allantois were used to verify accuracy of graft placement by demonstrating within each experiment that allantoic cells contributed to the allantois when placed
15 there. In addition, they provided additional data on the fate of the basal allantoic cells, and on the developmental potential of the allantoic mid-region and tip (see below).

When all three allantoic regions were placed into
20 the base of the allantois of a host, grafted allantoic cells were well-incorporated in the allantois and were sometimes distributed amongst two of the three allantoic regions delineated in this study: base, mid-portion and distal third (Fig. 5). Grafted cells were typically
25 well-spread across the girth of the host allantois, as demonstrated by their absence in many consecutive sections.

Of potentially important significance was our finding that when placed into the base of the allantois, donor allantoic cells from all three allantoic regions were conspicuously absent in the fetus, either as
5 integral tissue components or contained within the fetal vasculature. Rather, most donor allantoic grafts contributed cells only to the allantois or, in a minority of cases, to the yolk sac (Fig. 5; see below). Moreover, only 8.2% of all donor grafts contributed cells to the
10 base of the allantois. This observation suggests that normal cell movement within the allantois is from proximal-to-distal. Of the chimeras which contained a few donor cells in the base, colonization by these donor cells was found in the nascent allantoic vasculature
15 (data not shown).

In four chimeras, some donor allantoic cells contributed to the endothelium of the immediately adjacent omphalomesenteric artery (Fig. 5A, B). However, because the overwhelming majority of grafts contributed
20 exclusively to the allantois (45/49), we believe that contribution to the yolk sac vasculature may have been due to contamination of the yolk sac by grafted cells when the injection pipette was withdrawn from the conceptus, or possibly by displacement of part of the
25 graft to the yolk sac during subsequent culture. This is because in one instance we found a small cluster of unincorporated cells (chimera number 3, Fig. 5B) adherent

to the outside of the yolk sac at the level of the site of injection into the base of the allantois.

Some striking and potentially significant differences between the three allantoic regions were also evident from these grafts. In particular, although all three allantoic regions had been placed into the base of the allantois, cells derived from the donor mid-region and tip made significant contributions to the chorioallantoic fusion junction of the host (Fig. 8), with cells from the tip contributing the highest number. In contrast, donor cells from the base of the allantois rarely translocated that far. Also, the base of the allantois contributed the highest percentage of descendants to the allantoic endothelial lineage, and were found predominantly in the host allantoic mid-region (Fig. 8).

Fig. 8 diagrams colonization patterns by cell type of grafted allantoic tissue into host allantoises. The colonization patterns of donor allantoic tissue 23 hours after placement into the allantois of host conceptuses. The total number of grafted cells according to donor allantoic region was: base (5613), mid-portion (9538), and tip (2501).

Heterotopic Transplantations of Donor Allantoic Tissue

Prospective Paraxial Mesoderm (PM)

None of the three allantoic regions colonized pre-somitic or paraxial(somitic) mesoderm (Table 5B). In

the chimeras obtained from all three donor allantoic regions, donor colonization of the fetus at the level of the prospective paraxial mesoderm was again in the endothelial lining of the dorsal aorta and in mesenchyme adjacent to the dorsal aorta (Fig. 7). The allantoic mid-portion revealed the highest average number of grafted cells, whereas the base and tip contributed relatively few. Nevertheless, most chimeras containing grafts of the allantoic mid-portion also contained clumps of unincorporated tissue, suggesting that not all cells of the allantoic mid-region can colonize this embryonic region. Interestingly, the base of the allantois did not exhibit the relative pluripotency in this region of the conceptus that it did when transplanted into prospective LPM.

Discussion

We have investigated the developmental potential of the extraembryonic mesoderm of the headfold stage allantois. In addition, the fate of cells in the base of the allantois was followed for the first time. Under the conditions used, all fetal and extraembryonic lineages in the LacZ-containing donor conceptuses stained strongly blue upon X-gal staining, both before and after culture, with the exception of a few donor cells of the erythropoietic lineage, due to co-staining in benzidine.

During the headfold stage, the primitive streak has fully elongated from its posteriormost site at the embryonic/extraembryonic junction of the conceptus to the

distal tip of the egg cylinder; it is still an active source of mesodermal tissue at this stage (P.P.L. Tam and R.S.P. Beddington, supra, 1987). The developmental potency of the headfold-stage allantois was challenged by placing it into two sites of the primitive streak of synchronous embryos: the mid-portion of the primitive streak where mesoderm emerges as lateral plate mesoderm, and the primitive streak where paraxial mesoderm is formed, just posterior to the node (P.P.L. Tam and R.S.P. Beddington, supra, 1987). In addition, placement of the distal-third of the allantois into the base, originally intended to control for contribution of donor allantoic cells to the allantois in the second set of experiments (Table 4B), provided additional data on the growth and state of differentiation of the headfold-stage allantois.

The Allantois and Embryonic Mesoderm

All three regions of the donor allantois contributed predominantly to the endothelium of the dorsal aorta and adjacent mesenchyme when placed into the fetus. This mesenchyme is thought to be recruited into connective tissue and/or smooth muscle comprising the surrounding tunicae of the mature aorta (Arey, 1966; reviewed in Noden, 1989).

Only the base of the allantois exhibited a greater developmental potential than the other two regions when placed into prospective lateral plate mesoderm, contributing descendants to the endoderm of the future coelom as well as to surface ectoderm and lateral plate

mesoderm, similar to the orthotopic control grafts.

Thus, the base of the allantois may contain a somewhat more developmentally labile population of cells than the two distal allantoic regions. The relative pluripotency
5 of the base of the allantois is not surprising, given that cells in the base of the allantois are the freshest mesodermal cells to emerge from the posterior streak until the 3-somite stage (P.P.L. Tam and R.S.P. Beddington, supra, 1987), and therefore, they may be the
10 least differentiated.

The fetal lineages colonized by allantoic tissue following placement into the primitive streak at the level of prospective paraxial mesoderm revealed no overlap with the orthotopic control grafts. In
15 particular, none of the regions of the allantois contributed to paraxial mesoderm. This suggests that the allantois share few, if any, properties with presomitic, or paraxial, mesoderm. The base and the tip of the donor allantois exhibited the fewest number of grafted cells
20 per embryo after placement into prospective paraxial mesoderm whereas the mid-region produced the most descendants. This suggests that the environment at this level of the streak cannot support pluripotent basal cells and more specialized tip cells. It further
25 suggests that those grafted cells that have become angioblasts may have the best chance of becoming incorporated into this less "allantoic-favorable" region of the conceptus.

State of Differentiation of the Allantois Along
its Proximodistal Axis

Grafts of allantoic tissue into the base of the allantois confirmed that there appear to be significant differences in the state of differentiation along the proximodistal axis of the headfold-stage allantois. This was manifest in the final translocation profiles of the three allantoic regions after placement into the base of a host allantois (Fig. 5). The majority of basal allantoic cells moved only as far as the allantoic midregion. In contrast, the majority of donor cells whose origin was the allantoic mid-region or tip moved further distally. The tip exhibited the largest fraction of cells in the chorioallantoic fusion junction (Fig. 8). This suggests that allantoic tip cells might be specialized for chorioallantoic fusion as early as the headfold stage or that cells destined for the endothelial cell lineage at the fusion junction possess properties different from those in the mid-region. Allantoic tip cells probably do not express the entire repertory of genes required for chorioallantoic fusion at the headfold stage, however, because previous studies have shown that the distal half of the allantois is not competent to fuse with the chorion until it has attained developmental maturity, typically at the equivalent of 4-6 somite pairs (K.M. Downs and R.L. Gardner, supra, 1995).

Differentiation of Allantoic Mesoderm into
Angioblasts: The Position-Effect Model

Previous studies have suggested that all mesoderm has the ability to undergo vasculogenesis, given the
5 correct environment (Pardanaud, et al., 1987; Coffin and Poole, 1988). The heart, large blood vessels and the vitelline vasculature are formed by vasculogenesis, a uniquely embryonic process in which pluripotent mesodermal cells differentiate into angioblasts that
10 subsequently aggregate and assemble in situ into new blood vessels. Angiogenesis, which is the formation of blood vessels from pre-existing ones, occurs once the major vascular systems are in place.

Angiogenesis occurs during both embryogenesis and
15 throughout the life of the organism. Very little is known about how the umbilical vasculature is formed. Electron micrographic analyses of the allantois have not been presented in enough detail to confirm the existence of angiogenic clusters (Tamarin and Boyde, 1977), but
20 Ellington (1985) has described rudiments of the vascular system in the rat by 10 dpc (equivalent to approximately 4-somite pairs in the mouse). Expression studies have also shown that Vascular Endothelial Growth Factor (VEGF), its two receptors, VEGFR1-flt-1 and VEGFR2-flk-1
25 (Yamaguchi, et al., 1993; Breier, et al., 1995) and TEK (tie-2) (Schnurch and Risau, 1993) are expressed early in the allantois. These suggested that vasculogenesis was occurring in the pre-fusion allantois, but without examination of the developmental fate and potency of

allantoic cells, the true significance of these patterns could not be verified.

Our transplantation experiments support the possibility that the umbilical vasculature is formed by
5 vasculogenesis rather than by angiogenesis. This is because allantoic angioblasts were found incorporated in the large vessel endothelium of the dorsal aorta and intersegmental arteries rather than in small capillaries. Although it is possible that the nearby yolk sac
10 contributes endothelial cells to the allantois, making angiogenesis the principal mechanism of formation of the umbilical vasculature rather than *de novo* vasculogenesis, we have some evidence that this is not the case. Yolk sac transplantations demonstrated that the earliest time
15 at which yolk sac cells move into the allantois is after fusion, at about 10-somite pairs (M. Blahnik, S. Gifford, and K.M. Downs, unpublished data). This is approximately 22 hours after the headfold stage at which the donor allantoises used in this study were removed for
20 transplantation. Also, benzidine staining and cell profiles demonstrated that all of the yolk sac-derived cells within the host allantois appear to be red blood cells.

If angioblasts are formed *de novo* within the
25 allantois, then how do extraembryonic mesodermal cells that comprise the allantoic bud differentiate into angioblasts? Two lines of evidence produced here suggest that distance of pluripotent mesodermal cells from the

posterior streak dictates angioblast differentiation.

First, movement within the growing allantois is from proximal to distal. Second, distinct differences in the state of differentiation were discovered along the

5 proximodistal axis of the headfold-stage allantois.

Basal allantoic cells were relatively more pluripotent than cells in the distal allantoic region. The allantoic mid-region was most efficient at colonizing the endothelium of the fetus, producing the highest number of
10 chimeras and grafted cell descendants. Cells in the allantoic tip were most efficient at colonizing the chorioallantoic fusion junction.

Together, these data suggest that extraembryonic mesoderm destined for the allantois emerges from the
15 primitive streak in a relatively pluripotent state, but as it is pushed distally by sustained addition of nascent extraembryonic mesoderm into the allantoic base, it moves out of the sphere of influence of the primitive streak and differentiates into angioblasts. Movement farther
20 into the tip entails further differentiation, either into allantoic cells specialized in fusion with the chorion or possibly into specialized umbilical endothelial cells. This model, called the "Position-Effect Model" of angioblast formation, is described in Fig. 9.

25 Position-dependent differentiation has been previously described for the trophoctoderm (R.L. Gardner, et al., 1973; Copp, 1978). The distinct fates of mural and polar trophoctoderm may be a consequence of their

position with respect to the inner cell mass (ICM; R.L. Gardner and R.S.P. Beddington, supra, 1988). Close contact with the inner cell mass results in proliferation of polar trophectoderm and differentiation into
5 extraembryonic ectoderm whereas giant cells are formed when the mural trophectoderm is deprived of contact with the ICM. Then, once a critical distance from the ICM-derived primitive ectoderm is achieved by the polarmost trophectoderm cells, they differentiate into
10 secondary giant cells.

The Base of the Allantois and the Germ Line

Ozdzenski (1967) hypothesized that a small population of alkaline phosphatase-positive cells that resides in the base of the headfold-stage allantois
15 contributes either to the mature allantois, to the germ line or to both. Our experiments represent the first direct attempt to resolve this hypothesis by following the fate of cells in the base of the allantois.

Results presented here suggest that cells in the
20 base of the headfold-stage allantois contribute only to the mature allantois. None of the basal allantoic cells were found in the fetus. That the grafts had ample time to translocate out of the base of the host allantois was demonstrated by the observation that most grafted
25 descendants were found in the distal two-thirds of the host allantois at the end of the culture period.

Thus, these data suggest that the fate of cells in the base of the allantois is to contribute to somatic

lineages, e.g., the allantois, rather than to become incorporated into the future germ line (Ozdzinski, 1967). Nevertheless, the possibility that the base of the allantois contains the future germ line cannot be
5 entirely ruled out. One reason is that the triangle of allantoic base/primitive streak had not been used in the transplantations and the germ cells may lie in this small region (Fig. 3). The other is that the germ cells failed to survive transplantation. The latter
10 possibility seems somewhat unlikely, as previous studies have shown that cells taken from this region survive culture and contribute extensively to somatic chimeras (Matsui, et al., 1992). In one instance, these cultured cells even contributed to the germ line (Labosky, et al.,
15 1994). Therefore, definitive proof of the whereabouts of the PGCs before colonization of the hindgut awaits further analysis.

Conclusions

The main function of the allantois is to fuse with
20 the chorion and vascularize, thereby forming the umbilical component of the chorioallantoic placenta. Angioblasts appear almost as soon as the allantois emerges from the posterior primitive streak. Allantoic vascularization is not dependent upon fusion with the
25 chorion (T.P. Yamaguchi, et al., Development 118:489-498, 1993; K.M. Downs and R.L. Gardner, supra, 1995). Moreover, formation of the umbilical vasculature does not appear to be dependent upon interaction with endoderm.

Our discovery of the whereabouts of pluripotent and differentiated cells within the allantois provides, for the first time, a developmental blueprint essential for understanding the role of genes expressed in the allantois during the headfold-stage. The allantois, heretofore little studied, offers a promising and highly manipulable system to discover how angioblasts are formed from pluripotent mesoderm in the living mammalian conceptus.

10 Gene Targeting in the Murine Allantois

At the headfold stage of mouse development, angioblasts are present in the allantois. The Examples above describe a technique for culturing and transplanting headfold stage allantoic cells into the allantoises of host embryos. These donor cells incorporate into the developing vasculature. If allantoic angioblasts are genetically manipulable, transplantation of transfected angioblasts could be used for genetic studies of vascular development. This method might also prove useful for providing exogenous gene products for therapeutic uses in developmentally-compromised fetuses.

Mice of a modified ROSA26 strain (ROSA26*), transgenic for the lacZ gene, provided donor material for transplantation. A thin glass capillary punctured the yolk sac, and suction through the capillary was used to remove the elongated allantoises from headfold stage embryos. The removed allantoises were plated in culture

medium containing 50% rat serum and remained in culture overnight. An X-gal stained section from a headfold stage embryo of the ROSA26* donor strain demonstrated that all cells of the embryo, including the cells of the allantois, were positive for blue X-gal staining. Overnight cultures of donor allantoises were dissociated and injected into the allantoises of host embryos which had been cultured for 18 hours starting at headfold stage (see Fig. 2). Injected host embryos were returned to culture for a further 8 hours. Injections of the host embryos pierced the yolk sac at the site near the chorion which is free of vasculature.

At the end of the final incubation period, the injected embryos had developed to the somite stage. Eight hours after injection, the injected embryos were fixed in 4% paraformaldehyde. After fixation, embryos were stained with X-gal, embedded in paraffin, and sectioned at 4 μ m. Sections were then dewaxed and stained with nuclear fast red.

Results

Stained slides were examined to detect blue donor allantoic cells within the host embryos. We looked for blue cells on the yolk sac, amnion, fetus, and allantois. Incorporation of donor allantoic cells occurred almost exclusively in the allantois of the host. The incorporated cells appeared as endothelial, mesothelial, and mesenchymal cells.

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In examining the allantois, we divided it into four regions, to determine whether the transplanted allantoic cells incorporate preferentially into specific regions of the allantois. The four divisions consisted of: the base region close to the fetus, the mid-region, the distal region nearing the chorion, and the fusion junction, where the allantois is attached to the chorion. The donor cells incorporated into the host allantois predominantly in the distal and fusion junction regions. Very few incorporated into the mid region, and none were present in the base. Although clusters of donor cells did attach to the yolk sac and amnion, these cells did not incorporate into the structure of these membranes and remained attached only to the surface.

Tables 2-4 summarize our findings. Table 2 shows the number of incorporated donor cells found in each region of the host allantois. Table 3 shows the number of incorporated donor cells which we classified as each specific allantoic cell type. Table 4 combines these findings by recording the regional distribution of each cell type.

TABLE 2

Distribution of Incorporated Donor Cells

	Mid Region	Distal Region	Fusion Junction	Total
n = 10	2 (0.04%)	2794 (51.84%)	2594 (48.13%)	5390
Mean \pm SEM	0.2 \pm 0.2	279.4 \pm 181.7	259.94 \pm 72.0	

TABLE 3

Type of Incorporated Donor Cells

	Endothelium	Mesothelium	Mesenchymal	Total
n = 10	3039 (56.38%)	433 (8.03%)	1918 (35.58%)	5390
Mean \pm SEM	303.9 \pm 130.7	43.3 \pm 22.2	191.8 \pm 68.7	

TABLE 4

Distribution by Cell Type

	Endothelium	Mesothelium	Mesenchymal	Totals
Mid Region		2		2
Distal Region	1597	278	949	2794
Fusion Junction	1472	153	969	2594
Totals	3039	431	1918	5390

The above-described experiment showed that: (1) Allantoic cells were able to survive removal from the embryo, overnight culture, dissociation of the cultured allantois, and transplantation into a host embryo. (2) The surviving transplanted cells were able to incorporate into the host embryo. (3) Incorporation occurred primarily in the distal and fusion junction regions of the host allantois. Transplanted cells which adhered to the yolk sac or amnion did not appear to incorporate into these tissues. (4) In the allantois, the transplanted cells appeared as various cell types, including endothelial cells which would be expected to line the vasculature of the umbilical cord. Thus, these transplanted cells could potentially be useful for releasing the products of transfected genes into the bloodstream of a developing fetus. Genetic engineering

of these allantoic cells could also be useful for studying the function of various genes in normal vasculogenesis and in malfunctions of vasculogenesis.

II. In Vitro System of Vasculogenesis

5 Materials and Methods

i. Mouse strains

Two mouse strains provided embryonic material for this study and were maintained on a 12-hour light/dark cycle (dark period 13.00-1.00). Matings between the F1
10 generation of (C57BL/6 X CBA) provided non-transgenic host conceptuses. F1 females of the (C57BL/6 X CBA) strain mated with homozygous *lacZ/lacZ* males of similar genetic background (ROSA26*, G. Friedrich and P. Soriano, Genes Dev. 5:1513-1523, 1991; B.P. Zambrowicz, et al.,
15 Proc. Natl. Acad. Sci. USA 94:3789-3794, 1997; K.M. Downs and C. Harmann, supra, 1997; K.M. Downs, et al., supra, 1998) provided *lacZ*-expressing donor allantoises.

ii. Dissection, staging, and culture of conceptuses

20 Pregnant females were killed by cervical dislocation at 11.00 a.m. on the 8th day of gestation (approximately 7.75 dpc) or the following day at the same time (approximately 8.75 dpc). Conceptuses were dissected from implantation sites (K.M. Downs and R.L. Gardner,
25 Development 121:407-416, 1995), Reichert's membrane and associated trophoblast were reflected, and embryos were staged as previously described (K.M. Downs and T. Davies, Development 118-1255-1266, 1993) and as described in

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Brown (N.A. Brown, Postimplantation Mammalian Embryos: A Practical Approach, 1990). Normal headfold-stage conceptuses were cultured as previously described by Downs and Gardner (K.M. Downs and R.L. Gardner, supra, 1995) for up to 24 hours if they were to be used for immunohistochemistry or for up to 32 hours if they were to be used as hosts and control conceptuses in transplantation experiments. At the end of incubation, all cultured conceptuses were scored for the extent of morphological development as described in Brown (N.A. Brown, supra, 1990); those showing abnormal development were not used (3.0% were excluded due to stunted growth of the allantois; n=65).

iii. Culture of allantoises and dye exclusion

For survival and vascularization, allantoises were mouth-aspirated into a hand-pulled glass microcapillary (K.M. Downs and R.L. Gardner, supra, 1995; K.M. Downs, et al., supra, 1998) and either cultured in suspension as previously described (K.M. Downs, et al., supra, 1998) or placed individually (i) into wells of 24-well tissue-culture plates (Falcon 3047), (ii) onto glass cover slips (12 mm, Fisher) coated for 30 minutes in filtered (0.45 μ m, cellulose acetate) poly-D-lysine (1 mg/ml double processed tissue culture water; Sigma) inserted into wells of 24-well tissue-culture plates and rinsed 34 times with sterile water, (iii) into sterile 8-well plastic chamber slides (Lab-Tek, Nunc), or (iv) into 8-well glass chamber slides some of which were

coated in poly-D-lysine. Allantoises were cultured in 0.5 ml (24-well tissue-culture dishes or Falcon culture tubes (K.M. Downs and R.L. Gardner, supra, 1995) or 0.4 ml (8-well chamber slides) of culture medium (Dulbecco's modified Eagle's medium (DMEM) containing either equal parts of immediately-centrifuged and heat-inactivated rat serum as previously described (K.M. Downs and R.L. Gardner, supra, 1995) or 5, 10 and 20% heat-inactivated fetal calf serum (Gibco-BRL; frozen and thawed twice before using) for 24-72 hours at 37°C in 6.2% CO₂ in air. For culture longer than 24 hours, allantoises were given completely fresh medium at 24-hour intervals. In some experiments, erythrosin B (Fisher; 0.04% in phosphate-buffered saline (PBS); H.J. Phillips and J.E. Terryberry, Exp. Cell Res. 13:341-347, 1957) was diluted 1:1 in dissection medium to verify allantoic cell viability. Vascular Endothelial Growth Factor (R & D System,s Minneapolis, MN) was prepared according to the manufacturer's instructions and was added to culture medium containing 5% FCS in concentrations of 1-20 ng/ml.

DiI (DiI/DiIC18(3); Molecular Probes) labeling of the basal portion of the allantois was carried out in some experiments by dipping the bottom of the allantois into a solution of DiI (1 part 0.5% DiI in absolute alcohol: 9 parts 0.3 M sucrose) as it was held by mouth aspiration in a capillary of slightly smaller diameter. Following culture, DiI-labeled explants were fixed in 4%

paraformaldehyde for 1 hour, rinsed in PBS, and examined with fluorescent optics.

At the time of visualization, culture medium was replaced with dissection medium (K.M. Downs and R.L. Gardner, supra, 1995), and the morphology of the allantoises was noted in an inverted compound microscope (Nikon). Some allantoic cultures were fixed in 4% paraformaldehyde at 4°C for 30 minutes and stained with X-gal (J.R. Sanes, et al., EMBO J. 5:3133-3142, 1986) at 37°C for 12-15 hours.

iv. Dissociation and transplantation of cultured allantoic cells

For dissociation of plated allantoises, culture medium was removed by suction with a sterile Pasteur pipette, and the allantoises were rinsed once in sterile PBS (dissolved in tissue culture grade distilled water, Sigma). 0.5 ml of trypsin solution (0.05% trypsin, 0.53 mM EDTA, in Hanks' Balanced Salt Solution; Gibco BRL) was added to each well, and the culture plates were incubated at 37°C in 6.2% CO₂ for 10-20 minutes. Trypsin was inactivated by addition of 0.5 ml dissection medium. Allantoic tissue was triturated gently five times with a sterile 5 ml plastic pipette tip (Bio Rad) with the tip opening flamed smooth (I.D. approx. 1 mm). The contents of the wells were combined in a centrifuge tube, and spun in a clinical centrifuge at approximately 1000 revs/minute for 5-10 minutes. After centrifugation, the

supernatant was removed and allantoic cells were resuspended in dissection medium.

For each transplanted conceptus, 1 μ l of the donor cell suspension, which contained an average of 1042 cells (s.d. 435; n=4 allantoises), was measured onto a sterile tissue culture dish. This drop was mouth-aspirated into a thin hand-pulled glass capillary (I. D. approximately 60 μ m), which was then used to inject the donor tissue into a host conceptus that had been cultured overnight and whose own allantois was therefore at a similar stage. The yolk sac of the host conceptus was pierced by the glass capillary in the unvascularized region close to the chorionic membrane to avoid rupturing the yolk sac vasculature and, after piercing the mesothelium of the host allantois, the contents of the capillary were blown out into the interior of the host allantois. Injected host conceptuses were returned to culture for a further 8 hours to allow time for integration of donor cells, after which the fetuses of control and operated host conceptuses contained approximately 14-16-somite pairs. Donor cell contribution to the host was scored as previously described (K.M. Downs and C. Harmann, supra, 1997; K.M. Downs, et al., supra, 1998).

v. Histological preparation of conceptuses

Conceptuses were prepared for histological analysis (K.M. Downs and C. Harmann, supra, 1997; K.M. Downs, et al., supra, 1998). Briefly, all conceptuses were fixed in 4% paraformaldehyde for 2 hours at 4°C, and rinsed

three times in PBS. If they had been used in transplantation experiments, operated and control conceptuses were then stained with X-gal at 37°C for 12-15 hours (K.M. Downs and C. Harmann, supra, 1997), but if immunohistochemistry was used on lacZ-containing conceptuses, X-gal-staining was limited to 6 hours. All conceptuses were embedded in paraffin and sectioned at a thickness of either 4 μ m or, if they were used for immunohistochemistry, at 6 μ m.

vi. Immunohistochemistry

Immunohistochemistry was as previously described (K.M. Downs, et al., supra, 1998), with the exception that immunohistochemistry for VCAM-1 (C-19, Santa Cruz Biotechnology, Inc., cat. # SC-1504) in paraffin-embedded tissue included an unmasking step where, immediately after dewaxing, sections were placed in 10 mM SSC (T. Maniatis, et al., Molecular Cloning: A Laboratory Manual, p. 447, 1982), pH 6.0, followed by 1 minute of heating in the microwave to just below the boiling point. This was followed by 1 minute of cooling. Heating and cooling were repeated two times before the SSC was changed, after which the sections underwent five more cycles of heating, cooling and rinsing in SSC. After the last cycle, sections were placed in PBS in preparation for immunohistochemistry. The chromogen diaminobenzidine (DAB; Sigma) was used to detect binding of horseradish peroxidase-conjugated secondary antibodies in single antibody experiments. Experiments involving VCAM-1

initially included Flk-1 controls (Santa Cruz
Biotechnology, Inc. cat. #SC-315; K.M. Downs, et al.,
supra, 1998) and employed embryos older than 6-somite
pairs, as these allantoises were previously shown to
5 express VCAM1 (G.C. Gurtner, et al., supra, 1995; L.
Kwee, et al., supra, 1995). Once the staining pattern of
VCAM-1 was established for older embryos, Flk-1 was
omitted and older embryos were used in every experiment
to compare staining intensity of VCAM-1 in younger
10 allantoises. In experiments involving use of two
antibodies or a single antibody in conjunction with X-gal
staining, alkaline phosphatase (AP) was conjugated to the
secondary antibody and either Fast Blue BB or Fast Red TR
salts were used to detect the AP (K. Miller, Theory and
15 Practice of Histological Techniques, pp. 435-470, 1996).
Control slides were incubated in blocking serum without
antibody or in a solution of antibody prebound for 3
hours at room temperature with Flk-1 or VCAM-1 control
peptides (Santa Cruz Biotechnology, Inc.; K.M. Downs, et
20 al., supra, 1998). Allantoises that had been cultured on
plastic slides and immunostained were coverslipped in
aqueous mounting medium (Surgipath, Lerner Laboratories,
Pittsburgh, PA) and visualized in a compound microscope
(Nikon). Some material was counterstained with nuclear
25 fast red or hematoxylin, as described in the figure
legends.

vii. Uptake of DiI-Ac-LDL

To distinguish mature endothelial cells from immature endothelial cells, uptake by the allantoic cultures of acetylated low-density lipoprotein labeled with 1,1-dioctadecyl 1,3,3,3,3-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) was analyzed as described in Dubois, et al. (N.A. Dubois, et al., Exp. Cell Res. 196:302-313, 1991). Allantoises cultured for 24 or 72 hours (with feeding), as well as positive control endothelioma (E'oma) cells (J. Obeso, et al., Lab. Invest. 63:259-269, 1990) and negative control L929 fibroblasts (American Tissue Culture Collection; generous gifts from Professor Robert Auerbach, University of Wisconsin) were incubated with 10 µg/ml DiI-Ac-LDL for 12 hours at 37°C in DMEM containing 5% heat inactivated fetal calf serum or with control medium without DiI-Ac-LDL. The cultures were washed several times with PBS, fixed in 4% paraformaldehyde, and examined with a fluorescence microscope. Fluorescence was visualized using a standard rhodamine excitation/emission filter combination.

viii. Transfection assays

Headfold-stage allantoises were plated individually on 24-well tissue culture plates and cultured for 12 or 18 hours before transfection. At the time of transfection, culture medium was replaced with 0.5 ml heat- and gas-equilibrated DMEM containing 5% fetal calf serum (Gibco-BRL) and allantoises were transfected using

allantoises that had been maintained in unchanged culture medium were compared with allantoises that had been given fresh culture medium daily. At these time points, the morphology of the cultured allantoises was noted, and the diameter of the cultures measured. Cell counts were taken at 24 and 72 hours.

Fig. 10 describes the morphology and *lacZ* expression in plated headfold-stage allantoises. Figs. 10A-C describe the comparison of a single explanted allantois grown for 24 hours (Fig. 10A), 48 hours (Fig. 10B) and 72 hours (Fig. 10C) in a 24-well tissue culture plate containing an equal volume of rat serum and DMEM. Vascular channels (black arrow, Figs. 10A-D, F), coalesced clusters of cells (white arrow), and peripheral mesenchymal cells (curved arrow, Figs. 10A-C, F) are shown. (Fig. 10D): Donor allantoic explant of *lacZ*/+ genotype cultured for 20 hours and stained in X-gal. All cells were blue. (Fig. 10E): Explanted allantois cultured in 8-well glass chamber slide for 72 hours (and fed at 24 hour intervals) that did not maintain its vasculature (Table 5). (Fig. 10F): Explanted allantois grown for 24 hours in 24-well tissue culture plate in 5% fetal calf serum. Asterisk indicates central mass of undifferentiated cells. Scale bar in 10F: 300 μ m (10A-C); 72 μ m (10D); 240 μ m (10E); 310 μ m (10F).

At 24 hours, all plated allantoises had flattened out on the bottom of the dish and spread to form a circular mass of closely packed cells. They did not fill

the bottom of the culture well. All allantoises underwent vasculogenesis as evidenced by the development of conspicuous vascular networks, appearing as complex, criss-crossing channels throughout all but the
5 peripheral-most region of the allantois (K.M. Downs, et al., 1998; Fig. 10A; Table 5).

Coalesced clusters of cells were observed in association with vascular channels, and flattened cells were found in all spaces between the vascular channels.
10 Spindle-shaped fibroblast-like cells adherent to the plastic were visible at the perimeter of the circular allantoic mass. The vascular plexus and coalesced clusters expressed *flk-1* whereas the fibroblast-like cells on the periphery did not (K.M. Downs, et al., 1998;
15 Figs. 11A, B). Fig. 11 describes the immunohistochemistry used to localize Flk-1 and VCAM-1 in plated headfold-stage allantoises. Allantoises were explanted and plated individually in 8-well plastic chamber slides containing an equal volume of rat serum
20 and DMEM and cultured for 24 hours. Vascularized explants were prepared for immunohistochemistry as described in Materials and Methods. (Figs. 11A, B): Low and high magnification of anti-Flk-1-stained (brown) allantoises. (Figs. 11C, D): Low and high magnification
25 of anti-VCAM-1-stained (brown) allantoises. (Figs. 11E, F): Control allantoises minus primary antibody (Fig. 11E) and primary antibody prebound with control peptide (Fig. 11F). Tissue in all panels was counterstained with

hematoxylin. Scale bar in (Fig. 11F): 230 μm (Figs. 11A, C, E, F); 58 μm (Figs. 11B, D).

The majority, if not all, of the flattened cells in between the vascular plexus also appeared negative for
5 Flk-1 (K.M. Downs, et al., 1998; Fig. 11A, B). Counting at the end of 24 hours revealed an average of 5101 cells per allantois (3 experiments, n=29 allantoises).

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Condition	Substratum	Culture Medium (volume)	No. Expts	No. Plated	No. Survived	Vascularization		
						24 hours	48 hours	72 hours
1	Falcon plastic	50% rat serum (0.5 ml)	10	35	35 (100.0%)	Through out (100.0%)	Through out (100.0%)	Through out (94.3%)
2	poly-D-lysine coated glass cover slips	50% rat serum (0.5 ml)	3	16	16 (100.0%)	Through out (81.2%)	Through out (100.0%)	Through out (100.0%)
3*	poly-D-lysine-coated glass slides	50% rat serum (0.4 ml)	3	12	12 (100.0%)	Through out (83.3%)	Through out (75.0%)	Through out (50.0%)
4*	8-well glass slides	50% rat serum (0.4 ml)	3	10	10 (100.0%)	Through out (60.0%)	Through out (50.0%)	Through out (10.0%)
5*	8-well plastic slides	50% rat serum (0.4 ml)	3	10	10 (100.0%)	Through out (90.0%)	Through out (50.0%)	Through out (30.0%)
6	Falcon plastic	5% FCS (0.5 ml)	6	20	19 (95.0%)	Periphery [†] (100.0%)	None ^{††} (0.0%)	None ^{††} (0.0%)
7	Falcon plastic	10% FCS (0.5 ml)	3	7	7 (100.0%)	Through out (100.0%)	Breaking Down (71.4%) None ^{††} (28.6%)	None ^{††} (0.0%)
8	Falcon plastic	20% FCS (0.5 ml)	3	8	8 (100.0%)	Through out (100.0%)	Through out (87.5%) Breaking Down (12.5%)	Through out (12.5%) Breaking Down (62.5%) None ^{††} (25.0%)

Table 5. Comparison of vascularization of headfold-stage allantoises under different culture conditions. Allantoises were explanted and cultured (with feeding at 24-hour intervals) in DMEM-based media as described in the Table for 24-72 hours. All experiments included controls plated in 24-well dishes of Falcon plastic containing equal volumes of heat-inactivated rat serum and DMEM. The shaded rows indicated conditions most favorable to vascularization of explants.

* Allantoises that had not vascularized rounded up into plastic-adherent balls.

[†]The vasculature of average: to large-volume allantoises emanated from a central clump of undifferentiated cells (Fig. 1E), whereas vascularization in smaller explants occurred throughout, with no residue of undifferentiated cells in the explant's center.

^{††}Adherent fibroblast-like cells only were observed.

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By 48 hours, cultures that had been fed fresh medium at 24 hours had physically enlarged and reorganized (Table 5; compare Fig. 10A and B). In addition, cells on the periphery had spread and were no longer confluent with each other. In the absence of feeding, vascular channels had begun to break down (data not shown) and there was a trend toward smaller diameter in comparison with fed ones.

Feeding cultures at 48 hours resulted in significantly larger allantoises by 72 hours (data not shown), with an average number of 9129 cells per allantois (3 experiments, n=13 allantoises). The vasculature was intact and robust in the majority of explants (Table 5) though its appearance had again changed (compare Fig. 10B and C). Vascular channels were less extensive and the remaining vascular lumina were generally wider than observed at the previous two timepoints (compare Fig. 10A-C). Fed cultures maintained *flk-1* expression in the vascular channels at levels similar to those observed after 24 hours (data not shown). 18.2% of the unfed cultures retained vasculature, but others showed little (18.2%) or no (63.6%) vasculature (n=11 unfed allantoises). In the latter, much of the culture now consisted of fibroblast-like cells, with greater distances between cells on the periphery.

Allantoises removed from *lacZ*/+ transgenic conceptuses and cultured as described above, retained

5 X-gal activity throughout the entire culture period (Fig. 10D). However, as outlying cells were isolated from other cells and spread out more thinly on the plate, they were easily overfixed, which blocked X-gal staining (K.M. Downs and C. Harmann, supra, 1997). Fixation times of 10 or 30 minutes allowed blue staining throughout the cultures, including the outlying cells, whereas 90 minutes of fixation blocked staining only in the latter.

10 Survival and vascularization of explanted allantoises in different culture conditions

15 To determine whether the foregoing culture conditions were optimal for survival and differentiation of allantoic explants, we investigated growth and vascularization of explanted headfold-stage allantoises under several different conditions. In addition to tissue culture plastic, allantoises grew and vascularized for up to three days on poly-D-lysine coated glass cover slips (Table 5). However, allantoises fared less well in other conditions (Table 5). Although many allantoises initially vascularized in 8-well chamber slides, vascularization was not maintained over the 72-hour time period (Table 5). Where vascularity had been lost in cultures in chamber slides, allantoises rounded up into adherent balls (Fig. 10E).

25 Cell numbers at the end of the 24-hour culture period in explants grown in 5% FCS were slightly lower than those of allantoises grown in rat serum (4417 cells per allantois, 3 experiments, n=12 allantoises); further,

their gross morphological appearance was somewhat different (Fig. 10F). First, the flattened cells at the periphery of the allantoic mass presented a well-circumscribed, rather than diffuse, border. Second, in those allantoises that were initially of average or large volume, a Flk-1-positive vascular plexus had formed, but rather than criss-cross throughout the allantois, it emanated from a central core of morphologically undifferentiated cells (Fig. 10F).

Immunostaining revealed the presence of flk-1- and VCAM-1-expressing cells (data not shown), suggesting that the core comprised angioblasts and mesothelial cells.

Despite feeding, allantoises cultured in 5% FCS were typically devoid of vascular channels by 48 hours. By 72 hours, explants fed medium containing 5% FCS at 24-hour intervals contained an average number of 5649 cells per allantois in two experiments (N=5), and consisted predominantly of mesenchymal cells. Increasing the concentration of FCS to 10-20% FCS resulted in partial maintenance of vascular channels for up to 72 hours in only 12.5% of the explants. Finally, although explants cultured in 10% rat serum were indistinguishable from those cultured in 5% FCS, increasing the rat serum to 20% resulted in explants indistinguishable from those cultured in 50% rat serum (data not shown).

Thus, a high concentration of some factor(s) must be required for both formation and maintenance of endothelial cells in allantoic explants, either directly

or through maintenance of the mesothelium, previously
hypothesized to play a role in vascularization of the
core mesoderm (K.M. Downs, et al., supra, 1998). To test
that possibility, recombinant VEGF (1-10 ng/ml culture
5 medium) was added to explants at the start of culture in
5% FCS. Feeding at 24 hour intervals in the presence of
VEGF (2-10 ng/ml) resulted in retention of Flk-1
containing vascular channels and overlying VCAM-1
positive cells by 72 hours whereas untreated explants or
10 those treated with 1 ng/ml of VEGF were devoid of such
channels. Further cell survival was enhanced (78%
relative to the controls 36% in FCS alone).

Together these findings suggest that varying the
culture conditions of allantoic explants through serum
15 starvation or enrichment varies the state of the
endothelial cells, with low serum favoring formation of
non-epithelialized angioblasts, and high serum favoring
formation of endothelial channels. At least one of the
key growth factors required in formation and maintenance
20 of the vascularity appears to be VEGF. In addition,
there appears to be a strong correlation between co-
retention of endothelial and mesothelial cells.

Time course of allantoic vasculogenesis in
explant cultures

25 To discover whether headfold-stage allantoises
vascularized stereotypically in vitro, allantoises were
plated individually in 24-well tissue-culture dishes,
cultured in DMEM containing rat serum (1:1), and observed

at 4-hour intervals for up to 24 hours (Fig. 12). At 4 hours after plating, allantoises had attached to the plastic, but were little changed from their initial morphology, appearing just slightly larger and flattened but still retaining their elongated and tapered shape (Fig. 12A). At 8 hours, a small number of allantoises resembled those at 4 hours, but most were well-attached to the tissue culture plastic and exhibited fibroblast-like cells spreading out from the allantois (Fig. 12B). By 12 hours, all allantoises were well-adhered to the plastic and most were still somewhat elongated. At one end of the allantoic projection, cells were beginning to vascularize whereas at the opposite end, cells were piled up and appeared morphologically undifferentiated as described above (Fig. 12C). Labeling the basal end with DiI prior to plating confirmed that the latter was the base of the allantois (data not shown).

At 16 hours, all allantoises were well-vascularized, with criss-crossing vascular channels almost fully obscuring the few cells still piled up at the basal end of the allantois (Fig. 12D). Allantoises cultured for 20 hours (Fig. 12E) were indistinguishable from those at 24 hours (compare Figs. 12E and 10A; also, K.M. Downs, et al., supra, 1998). The flattened allantoic mass was nearly perfectly round by this time and contained a regular criss-crossing vascular plexus in all but the peripheral-most regions, which was populated by a single

layer of mesenchymal cells. DiI labeling revealed that cells from the basal region became incorporated into the vasculature, as well as into the overlying mesenchymal and attached mesothelial cell populations (date not shown). Thus, allantoises in isolation culture appear to differentiate stereotypically, and maintain the distoproximal gradient of differentiation (K.M. Downs and C. Harmann, supra, 1997; K.M. Downs, et al., supra, 1998).

10 Transplantation of cultured donor lacZ/+
 allantoic cells into host conceptuses

Transplantation was used to determine whether allantoic cells cultured for 24 hours were able to appropriately colonize host allantoises. Following dissociation, a portion of the donor cells was counted, some were replated to ensure viability and retention of lacZ-expression following dissociation, and others were transplanted into the allantoises of host conceptuses.

A total of 30 headfold-stage host conceptuses was cultured for 24 hours in three experiments. Two of 30 hosts were discarded, as they contained allantoises that had not fused with the chorion by the end of the culture period and were therefore judged abnormal. In the remaining 28 conceptuses, the yolk sac in the unvascularized region close to the chorion was pierced with a glass capillary containing donor cells (Fig. 13A) which was then directed into the host allantois (Fig. 13B) and the donor cells were gently blown in. Injected

host conceptuses were returned to culture for 8 hours to allow time for integration of donor cells, after which all 28 operated host conceptuses (Fig. 13C) were judged chimeric following X-gal staining (range of incorporated cell fragments 5-2239).

All injections resulted in donor cell integration into host allantoises. Colonization of donor allantoic cells was confined to the distal region and fusion junction of the host's allantois (Fig. 13D, E) and occurred with similar frequencies (data not shown). Fig. 13 describes transplantation of cultured donor *lacZ*/*+* allantoic cells into cultured host conceptuses. (Fig. 13A): 8.75 dpc cultured benzidine-stained (K.M. Downs and C. Harmann, supra, 1997) host conceptus viewed from the region of the ectoplacental cone to show the vessel-free region in the yolk sac (white arrow) selected for injection of donor cells into the allantois. (Fig. 13B): Schematic diagram of cultured 8.75 dpc conceptus in sagittal orientation showing the distal site of injection of donor cells into the host allantois. (Fig. 13C): Operated 8.75 dpc host conceptus cultured for a further 8 hours, viewed following removal of its yolk sac. (Fig. 13D): Schematic diagram of the fetus in Fig. 13C showing the regions of the allantois used to score the location of blue donor cells. (Fig. 13E): Cultured *lacZ*/*+* blue donor cells are visible in the distal (d, white arrowhead) and fusion junction (f, black arrowhead) regions of the host allantois. Insert: A higher

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magnification view of donor cells in the chorioallantoic
fusion junction shows incorporation of donor cells in the
host endothelium. (Fig. 13F): A clump of unincorporated
donor cells (arrow) in contact with, but not integrated
5 into, the host yolk sac. Conceptuses in (13E, F) were
counterstained in Nuclear Fast Red. Referring to Fig.
13, abbreviations are: al, allantois; b, brain; ch,
chorion; h, heart; p, placenta; s, somites; ys, yolk sac.
Scale bar in (Fig. 13F): 700 μ m (Figs. 13A, C); 200 μ m
10 (Fig. 13E); 70 μ m (Fig. 13E, insert); 50 μ m (Fig. 13F).

Fig. 14 plots the average number of donor *lacZ*/+
allantoic cells incorporated into host allantoises
according to cell type. Mean number of donor cell
fragments that incorporated into the host allantois were
15 classified as endothelial, mesothelial or mesenchymal
cells. Donor cells that had integrated ectopically (yolk
sac or amnion) are also indicated.

The incorporated cells appeared as three types (Fig.
14): the majority were endothelial, the fewest
20 representatives were found in the mesothelium, and others
were solitary mesenchymal-like cells, not part of the
endothelium or mesothelium. A few ectopically
incorporated cells were found in the yolk sac and amnion
and were limited to the mesothelial cell layer (Fig. 14).
25 Donor cells were never found integrated in the fetus. A
very small number of allantoic red blood cells was blue
but, as reported previously, these were assumed to be

host erythroid cells that had become associated with donor endothelium (K.M. Downs, et al., supra, 1998).

5 The average number of incorporated cells over three experiments was 31% of the total estimated number of cells that had been injected. 34% of injected cells were found as unintegrated clumps within the host conceptuses. Of these, approximately half were located within the allantois. The others were located either (i) free in the exocoelomic cavity, (ii) associated with but not
10 adherent to the yolk sac or amnion in the exocoelom (Fig. 13F), (iii) within the ectoplacental cone, or (iv) within the lumen of the gut. In the last category, donor cells had likely become adherent to the outer surface of the conceptus during injection and were internalized as the
15 gut formed. Based on these calculations, we conclude that the remaining 35% of injected cells were likely lost to the dissection medium during transplantation.

As a prelude to determining the Flk-1 status of donor cells in chimeric hosts, *flk-1* expression was
20 compared in freshly-recovered conceptuses (approximately 12-somite pairs) and headfold-stage conceptuses cultured to at least 12-somite pairs. We had previously reported that allantoic maturity, i.e., competence of the allantois to fuse with the chorion, appears to be under
25 the control of an internal timing mechanism which correlates with the number of somite pairs contained in the fetus (K.M. Downs and R.L. Gardner, supra, 1995; reviewed in K.M. Downs, supra, 1998). Headfold-stage

embryos cultured for 23-24-hours contain an average of 12-somite pairs at the culture endpoint (K.M. Downs and C. Harmann, supra, 1997; K.M. Downs, et al., supra, 1998); thus, headfold-stage allantoises cultured for 24
5 hours are considered to be the equivalent of intact allantoises of hosts containing approximately 12-somite pairs.

Fig. 15 illustrates localization of Flk-1 in allantoises of freshly-recovered, cultured and cultured
10 chimeric conceptuses. Allantois from (Fig. 15A) a freshly-recovered 8.75 dpc conceptus (approximately 14-16-somite pairs) and (Fig. 15B) a headfold-stage conceptus cultured for 24 hours (approximately 14-16 somite pairs) show *flk-1* expression (brown staining) in
15 the nascent vasculature (closed arrows). (Fig. 15C): Chimeric host conceptus containing integrated cultured *lacZ*/+ donor cells at the chorio-allantoic fusion junction. Black arrowheads indicate those blue donor *lacZ*/+ cells as a result of reaction with X-gal that
20 contain Flk-1, red as a result of reaction with alkaline phosphatase/Fast Red TR salt. White arrow indicates donor cells that are integrated in the mesothelium (and Flk-1) or clumped. (Fig. 15D): Higher magnification view of doubly-stained X-gal/Flk-1-positive donor cells
25 (black arrowheads). White arrow as in (Fig. 15C). Conceptuses in Figs. 15A and B were counterstained in hematoxylin. Abbreviations as in Fig. 13. Controls for X-gal staining were as previously described (K.M. Downs

and C. Harmann, supra, 1997); controls for Flk-1 in both cultured and ex vivo control conceptuses for all panels were as previously described (K.M. Downs, et al., supra, 1998). Scale bar in (Fig. 15D): 53 μ m (Figs. 15A, B and C); 12 μ m (Fig. 15D).

In both ex vivo and cultured conceptuses, Flk-1 was localized to the allantoic vasculature and was not expressed in mesothelium (Fig. 15A, B). In operated chimeric hosts, many, but not all, donor cells that had incorporated into the host's allantoic vasculature exhibited Flk-1 (Fig. 15C, D). None of the donor cells that had integrated into the mesothelium contained Flk-1, whereas clumps of unincorporated cells contained both Flk-1-positive and Flk-1-negative cells.

15 DiI-Ac-LDL uptake

The ability of cultured allantoic cells to take up DiI-Ac-LDL was examined to obtain further evidence that the Flk-1-positive cells were endothelial, with the caveat, however, that many (J.F. Nagelkerke, et al., J. Biol. Chem. 258:12221-12227, 1983; Voyta, et al., 1984; J.D. Rone and A.L. Goodman, supra, 1987; Z. Yablonka-Reuveni, Dev. Biol. 132:230-240, 1989; N. Chung-Welch, et al., Differentiation 42:44-53, 1989; N.A. Dubois, et al., supra, 1991; T. Sakamoto, et al., Curr. Eye Res. 14:621-627, 1995), but not all (J.D. Rone and A.L. Goodman, supra, 1987; J. Plendl, et al., Anat. Embryol. 194:445-456, 1996), endothelial cells take up DiI-Ac-LDL.

Fig. 16 illustrates uptake of DiI-Ac LDL. Light- (Figs. 16A, C and E) and fluorescent- (Figs. 16B, D and F) micrographs of E'oma cells (Figs. 16A and B), L929 fibroblasts (Figs. 16C and D) and headfold-stage allantois plated for 72 hours (Figs. 16E and F), all exposed to acetylated-DiI-low density lipoprotein for 12 hours as described in Materials and Methods.

Allantoises cultured for 24 and 72 hours (3 experiments per time point, ≥ 2 allantoises per experiment), as well as positive (E'oma) and negative (L929) control cell lines, were exposed to DiI-Ac-LDL for 12 hours. After removal of the excess DiI-Ac-LDL, cultures were examined for fluorescence. As expected, the control E'oma cells did show uptake of the fluorescent label (Figs. 16A and B), whereas L929 fibroblasts did not (Figs. 16C and D). All allantoic cultures treated in the presence of DiI-Ac-LDL showed no fluorescent labeling, either in the vascular channels or in the surrounding cells (Figs. 16E and F).

Expression of VCAM1 in intact and explanted allantoises

VCAM-1 is required in mesothelium for fusion of the allantois with the chorion (G.C. Gurtner, et al., supra, 1995; L. Kwee, et al., supra, 1995; J.T. Yang, et al., supra, 1995) and it is the only gene whose protein product has thus far been clearly demonstrated in allantoic mesothelium. As a prelude to investigating the presence and whereabouts of mesothelium in cultures of

explanted allantoises, spatiotemporal expression patterns of VCAM1 were analyzed in whole ex vivo conceptuses (headfold-to-8-somite pair stages).

Fig. 17 describes the spatiotemporal appearance of VCAM-1 in freshly-recovered conceptuses. Freshly-recovered conceptuses prepared for immunostaining against VCAM-1. (Fig. 17A): Allantois at late headfold-stage (LHF) showing one VCAM-1-positive cell (brown) in the putative mesothelium (white arrowhead). Black arrowheads indicate mouse hairs in cross-section inserted as fiducial markers for experiments not described in this application. (Fig. 17B): Allantois at 4-somite pairs (4-s) showing VCAM-1-positive mesothelium (white arrowhead) and VCAM-1-positive core cells (white arrow). (Fig. 17C) 6-somite pair (6-s) allantois. (Fig. 17D) 8-somite pair (8-s) allantois. Arrowheads and arrows as in (Fig. 17B). (Fig. 17E) 8-somite pair control conceptus without antibody (-Ab). (Fig. 17F) 8-somite pair control allantois in which antibody to VCAM-1 was prebound with control VCAM-1 peptide (+cp). All conceptuses were counterstained in hematoxylin. Abbreviations as in Fig. 12; am, amnion; ps, region of the primitive streak. Scale bar in (Fig. 17F): 40 μ m (Fig. 17A); 78 μ m (Fig. 17B); 110 μ m (Fig. 17C); 120 μ m (Figs. 17D, E and F).

VCAM-1 was not detected in late neural plate stage allantoises. At headfold stages, up to half of the distal allantois contained a flattened exterior layer of putative mesothelial cells (K.M. Downs, et al., supra,

1998) but expression of *VCAM1* was found in just a few
outer distal cells in a small number (33.3%) of
allantoises (Fig. 17A). However, between 2- and 8-somite
pairs, *VCAM1* expression spread proximally in the
mesothelium of all allantoises (Figs. 17B-F). Initially,
levels of VCAM-1 were modest but increased with allantoic
age, the most lightly staining cells always the most
proximal. By 6-8-somite pairs, levels of VCAM-1 were
highest throughout all but the mesothelium in the basal
region, which appeared negative in all allantoises at all
developmental stages (Figs. 17C-F). Some individual
mesothelial cells within largely VCAM-1-positive tracts
were lightly stained or negative at all developmental
stages examined.

In addition to mesothelium, VCAM-1 was found in core
allantoic mesoderm. Expression in the core was, however,
delayed with respect to the overlying mesothelium, first
being detectable at 3-4 somite pairs (Fig. 17B). At
these and all stages examined thereafter, levels of
VCAM-1 in the core were similar to those found in the
immediately suprajacent mesothelium and extended only as
far as the VCAM-1-positive mesothelium extended
proximally (Figs. 17B-D).

Fig. 18 illustrates simultaneous localization of
Flk-1 and VCAM-1 in intact and suspended allantoises.
Freshly recovered conceptus containing 14-somite pairs
(Fig. 18A) and headfold-stage conceptus cultured to 14-
somite pairs (Fig. 18B) were prepared for

immunohistochemistry as described above and immunostained with antibodies against Flk-1 (blue) and VCAM-1 (brown). White arrowheads, VCAM-1-positive mesothelium; white arrows, VCAM-1-positive core allantoic cells associated with the Flk-1-positive vascular plexus. (Fig. 18C): Explanted headfold stage allantois cultured in suspension for 24 hours and doubly-immunostained as described in (Figs. 18A and B). The proximal region is indicated by the horizontal bar beneath the explant. m, mesothelium; c, core allantoic cells. Scale bar in (Fig. 18C): 160 μ m (Figs. 18A and B); 88 μ m (Fig. 18C).

Double immunohistochemistry with Flk-1 revealed that VCAM-1-positive core cells were associated with but exclusive of the Flk-1-positive vasculature in both freshly-recovered and cultured conceptuses (12-16 somite pairs; Figs. 18A, B). VCAM-1-positive core cells were never associated with the vasculature at the base of the allantois (Figs. 17, 18). In addition, cells that contained neither Flk-1 nor VCAM-1 were scattered throughout allantoises.

We next looked for the presence of mesothelium in explanted allantoises and determined its topographical relationship to the allantoic vasculature by immunostaining with antibodies against VCAM-1 and comparing this staining pattern with that of Flk-1 (Fig. 11). By altering the focal plane during light microscopy, we found that VCAM-1-positive cells were distributed on top of the vascular plexus in explanted

allantoises (Fig. 11C-F). The peripheral fibroblast-like cells appeared negative for VCAM-1 (Fig. 11C) as they were for Flk-1 (Fig. 11A). Thus, allantoic mesothelium was present in culture in close association with the vasculature.

To provide further evidence that mesothelium survived culture, allantoises were suspended and cultured, rather than plated. We had previously shown that, in suspension, allantoises round up, and consist of an inner core of mesoderm encapsulated by a rind of putative mesothelium (K.M. Downs, *et al.*, *supra*, 1998). Here, double immunohistochemistry with antibodies against both VCAM-1 and Flk-1 was applied to suspended allantoises. We found that the outer rind of these was strongly VCAM-1-positive (Fig. 18C). Intriguingly, a short continuous tract of rind was VCAM-1-negative, which we interpreted as equivalent to the negative basal region of intact allantoises (Figs 17C, D and 18A, B). A hair placed in the base of the allantois prior to culture and immunostaining provided support for this interpretation, as all cells in the region of the hair were VCAM-1-negative. In addition, VCAM-1-positive core cells were found in association with the Flk-1 vasculature at the presumptive distal pole of suspended allantoises (Fig. 18C). Together, these results provided further support that mesothelium was present in culture in close juxtaposition to the vasculature.

Transfection of plated allantoises before and
after vascularization

On the basis of gene expression in intact allantoises and morphology, we conclude that at least three major cell populations are present in explanted allantoises: Flk-1-positive endothelial cells of the vasculature, VCAM-1-positive mesothelial cells overlying the vasculature, and a peripheral population of flattened mesenchymal cells that express neither *flk-1* nor *VCAM1*. Downs, et al. have recently proposed that allantoic cells might be an attractive vehicle for the delivery of therapeutic factors to the fetal blood circulation in order to ameliorate certain developmental defects (K.M. Downs, supra, 1999). Thus, to determine whether any of the three allantoic cell types could take up and express exogenous DNA, allantoises were transfected via the calcium phosphate precipitate method (F.L. Graham and A.J. van der Eb, supra, 1973) with a plasmid containing enhanced GFP expressed from the human cytomegalovirus early promoter.

Allantoic explants that had been cultured for 12 and 18 hours were exposed to the DNA precipitate for 6 hours. Shorter exposure times of 1, 2 and 4 hours resulted in healthy cultures but fewer transfectants, and an exposure time of 8 hours resulted in optimal numbers of transfectants but more dead cells (data not shown). Immediately after transfection, fresh DMEM-containing rat serum replaced 5% fetal calf serum and was also fed to

the absolute controls, and all cultures were returned to the incubator for a further 20-24 hours.

Transfected cells were scored (Table 6). The only cells that took up and expressed GFP in both the 12- and 18-hour cultures were the peripheral fibroblast-like cells that were adherent to the bottom of the dish (Fig. 19). Fig. 19 describes expression of GFP in plated allantoic explants. (Fig. 19A): Low magnification fluorescence view of GFP-expressing adherent mesenchymal cells in an allantoic explant that had been cultured for 12 hours prior to transfection. Expressing cells are green (white arrows) and are localized to the periphery of the explant. (Fig. 19B): Higher magnification of GFP-expressing mesenchymal cells (white arrows) from (19A). (Fig. 19C): Simultaneous bright field/fluorescent view of those GFP-expressing mesenchymal cells from (Fig. 19B) (white arrows) to show that they are distinctly different from cells contained within endothelial cell channels (black arrow). Scale bar in (Fig. 19C): 500 μ m (Fig. 19A); 50 μ m (Fig. 19B, C).

In the younger cultures, most of these cells were located at the explant periphery (Fig. 19) but, in older cultures, many adherent cells beneath the vasculature were also positive (data not shown). Plated allantoises exposed to the precipitate at both 12- and 18-hours after explantation took up DNA with similar frequencies (5.5% versus 6.0%, respectively, Table 6). The vasculature and

overlying mesothelial cells contained no detectable GFP,
suggesting that allantoic angioblasts (pre-endothelial
cells), endothelial and mesothelial cells are either
refractory to uptake of DNA by this standard method of
5 transfection or, if they can take it up, are unable to
express it. In addition, fewer cells were found on
average in transfected cultures as compared with the
controls (Table 6), suggesting that some allantoic cells
may be sensitive to calcium phosphate.

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No.	Number Cells/Allantois Early Vascularization (number of allantoises)				Number Cells/Allantois Post Vascularization (number of allantoises)			
	Control	FCS	Transfected	% Transfection Efficiency	Control	FCS	Transfected	% Transfection Efficiency
1	7933 (3)	6240 (3)	2375 (8)	7.3	2760 (3)	3083 (3)	3850 (10)	3.4
2	5375 (2)	2313 (2)	1358 (2)	7.7	3910 (2)	4252 (2)	2814 (8)	11.3
3	6210 (3)	4550 (3)	5145 (3)	1.4	6460 (3)	7700 (3)	3635 (9)	3.4
Average	6506 (8)	4368 (8)	2959 (13)	5.5	4377 (8)	5012 (8)	3433 (27)	6.0

Table 6. transfection efficiency of headfold-stage allantoises cultured for 12 and 18 hours. Headfold-stage allantoises were explanted onto 24-well tissue culture plates and cultured for 12 and 18 hours. at which time they were transfected for 6 hours with a plasmid containing GFP. The percent transfection efficiency was calculated as the total number of GFP-expressing cells over the total number of allantoic cells per treatment group.

Discussion

Characterization of allantoic explants

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In the Examples above, we have demonstrated that explanted murine allantoises cultured in a high
5 concentration of rat serum rapidly and reproducibly undergo vasculogenesis. Morphological vascularization was evident as early as 12 hours after plating onto tissue culture plastic. With feeding, most allantoises proliferated and maintained a Flk-1-positive vasculature
10 for at least 72 hours.

Cultured allantoises contained four identifiable cell types, angioblasts, endothelial, mesothelial and mesenchymal cells, all of which were normally present in intact allantoises of whole conceptuses. Use of
15 antibodies to illuminate the nature of these cells was validated by determining the location of Flk-1 and VCAM-1 in intact allantoises of both freshly-recovered and cultured conceptuses. We found that angioblasts were present as unvascularized Flk-1 cells in explants
20 cultured in low serum; VEGF promoted their survival and morphogenesis into vessels. Endothelial cells were present in both plated and suspended allantoises as Flk-1-containing vascular channels. Mesothelium was identified by expression of VCAM1 in cells that overlay
25 the vasculature; its presence in the cultures is significant because mesothelium is thought to provide signals required for vascularization of core mesoderm (K.M. Downs, et al., supra, 1998). Mesenchymal cells

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were classified on the basis of several criteria: (i) they were present as spindle-shaped adherent cells at the periphery of the explants, (ii) they did not express *flk-1* or *VCAM1*, (iii) they survived and proliferated in low serum, and (iv) some of them took up, incorporated and expressed GFP via calcium phosphate precipitation. The presence of mesenchyme beneath the necrotic vasculature in low serum also suggested that these cells underlie the explants throughout the entire culture period. Thus, we have established that the gross topographical relationships between cell populations in allantoic explants from top to bottom are: mesothelium, endothelium, and mesenchyme.

In addition to the foregoing results, appropriate integration of cultured allantoic cells into host allantoises following transplantation suggested that normal allantoic phenotype is maintained in culture. The majority of colonizing donor cells were endothelial and some also expressed *flk-1*; the fewest were mesothelial, and the others were mesenchymal, neither part of the vasculature nor the mesothelium. Because cultured allantoic cells retained *lacZ* activity for up to 72 hours, and allantoises have previously been shown to maintain *lacZ* expression at all developmental stages examined (K.M. Downs and C. Harmann, supra, 1995; K.M. Downs, et al., supra, 1998), loss of approximately one-third of injected cells during transplantation was likely due to leakage from the site of injection as cells

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were blown into the allantois rather than to loss of lacZ expression. Loss of another third of injected cells by clumping at the site of integration into the host was likely due to sub-optimal trypsinization (10 minutes rather than 20 minutes) in an effort to ensure cell viability rather than loss due to spurious differentiation because disruption of the stereotypic organization of the allantoic cultures was never observed. Alternatively, 8 hours may have been inadequate to ensure integration and maximal donor cell dispersal following transplantation. Culture periods beyond 36 hours were not used because global tissue necrosis had previously been observed despite feeding and culture of conceptuses individually (S. Gifford and K.M. Downs, unpublished observations).

Although initially favorable to vasculogenesis, conditions that employed 8-well chamber slides did not maintain vascularization in the explants despite feeding. This observation was not further explored, but one explanation may be that the optimal surface area: volume ratio of medium in these culture vessels had not been achieved. Although vasculogenesis took place in 5% FCS, the ensuing vasculature was neither extensive nor retained beyond 24 hours. Increasing concentrations of serum were only a partial solution, and did not enable adequate maintenance of the vasculature for 72 hours. We conclude from this that a high concentration of some serum factor(s) must be required for both formation and

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maintenance of endothelial cells in allantoic explants.
The identification of that factor and its normal site of
production in the conceptus are not, however, known.

5 The role of VCAM1 in differentiation and
 function of the murine allantois

 VCAM-1, originally isolated as a cytokine-inducible
adhesion molecule in human umbilical vein endothelial
cells (L.C. Osborn, et al., Cell 59:1203-1211, 1989; M.
Elices, et al., Cell 60:577-584, 1990; reviewed in L.
10 Kwee, et al., supra, 1995) is required, along with its
counter-receptor, $\alpha 4$ integrin, for chorioallantoic fusion
(G.C. Gurtner, et al., supra, 1995; L. Kwee, et al.,
supra, 1995; J.T. Yang, et al., supra, 1995). $\alpha 4$
integrin is expressed in the chorion, and VCAM1 in the
15 distal allantois, though the expressing cell types were
not identified (J.T. Yang, et al., supra, 1995; L. Kwee,
et al., supra, 1995; G.C. Gurtner, et al., supra, 1995).
Following chorioallantoic attachment, the allantoic
vasculature spreads onto the chorion and penetrates the
20 chorionic disk and labyrinth (K. Theiler, The House
Mouse, 1989). The role, if any, of VCAM-1 and $\alpha 4$ -
integrin in events other than attachment is, however, far
from clear.

 VCAM-1 was first detectable in a few distal
25 mesothelial cells at the headfold stage, but not earlier.
Expression of VCAM1 in mesothelium was then observed in
all allantoic stages thereafter, spreading proximally and
increasing over time. Elevated levels of VCAM-1 agree
with our previous prediction that the rate of

chorioallantoic fusion would be dependent upon increasing levels of a required adhesion factor during allantoic maturation in the exocoelom (K.M. Downs and R.L. Gardner, supra, 1995; K.M. Downs, supra, 1998). In the allantoic core, VCAM-1-containing cells were intimately associated with but distinct from Flk-1-positive endothelium. A role for these cells in endothelial support is unlikely, as they were not found associated with the Flk-1 vasculature in the basal region. One intriguing possibility is that the VCAM-1-containing core cells mediate vascularization of the chorionic and labyrinthine layers via VCAM-1 interactions, pulling the vasculature along as chorioallantoic adhesion proceeds.

Finally, our observation that expression of *VCAM1* begins in the distal most region of the allantois at the headfold stage supports previous tests of developmental potency in headfold-stage allantoises (K.M. Downs and C. Harmann, supra, 1997). When placed into the primitive streak of host fetuses at the level of emerging lateral plate mesoderm, distal donor allantoic cells were often extruded from rather than integrated into the host. In light of the spatiotemporal expression patterns of *VCAM1*, poor colonization was likely due to cell surface incompatibilities related to *VCAM1*, which is not expressed in the posterior region of the fetus (G.C. Gurtner, et al., supra, 1995; L. Kwee, et al., supra, 1995; this study, data not shown). The few distal donor allantoic cells that integrated into host blood vessels

likely did so as a result of their Flk-1 status and, therefore, endothelial nature.

5 Taken together, our results provide strong evidence that allantoic explants are a new and practical system for the study of vasculogenesis. Given the apparent lability of the vascular plexus to undergo rearrangement over 72-hours in culture, allantoises cultured *in vitro* may also shed light on the process of vascular remodeling. Lastly, the ability of allantoic cells to be
10 genetically-manipulated and to colonize the developing allantois may prove therapeutically valuable for *in utero* gene therapy in cases where a blood-borne circulating factor might ameliorate or cure certain fetal defects.

CLAIMS

I claim:

1. A method of fetal gene therapy comprising:
 - a) identifying a fetus with a genetic defect;
 - 5 b) obtaining donor allantois or umbilical cord cells compatible with the fetus, wherein the cells express a gene product that ameliorates the genetic defect; and
 - c) exposing the fetus to the allantois cells
- 10 of step (b), wherein a chimeric allantois/umbilical cord capable of supplying the gene product to the fetus is created.
2. The method of claim 1 wherein the donor cells are taken from the base of a donor allantois.
3. The method of claim 1 wherein step (c) comprises transplanting the cells to a region of the allantois/umbilical cord wherein an appropriate developmental environment is present.
4. The method of claim 1 wherein the allantois cells are taken from the mid-region of a donor allantois.

5. A method of gene therapy comprising
a) identifying a fetus with a genetic defect;
b) obtaining allantois cells compatible with
the fetus, wherein the cells express a gene product that
ameliorates the genetic defect; and

c) transplanting the allantois cells to the
fetus, wherein the transplanted cells develop into
endothelial cells which line the vasculature of the
umbilical cord and release the gene product into the
bloodstream of the fetus.

6. The method of claim 5 wherein the cells are
taken from the mid-level of a donor allantois.

7. The method of claim 5 wherein the cells are
taken from the base of a donor allantois.

8. The method of claim 5 wherein the cells are
taken from a dissociated cultured donor allantois.

9. The method of claim 5 wherein the cells
comprise a marker gene.

10. A method of fetal gene therapy comprising:
 - a) identifying a fetus with a genetic defect;
 - b) obtaining allantois or umbilical cord cells compatible with the fetus, wherein the cells
5 express a gene product that ameliorates the genetic defect and wherein the cells are capable of colonizing a fetal organ; and
 - c) exposing the fetus to the cells of step (b), wherein a chimeric fetal organ capable of supplying
10 the gene product to the fetus is created.
11. The method of claim 2 wherein the organ is liver.
12. The method of claim 2 wherein the method of exposure is microinjection into the fetal organ.
13. The method of claim 2, wherein the organ is the aorta.
14. A method of observing vasculogenesis comprising the steps of preparing an isolated allantois culture and observing vasculogenesis.
15. A transgenic allantois cell.

16. A method of delivering molecules to a fetus comprising:

a) obtaining donor allantois or umbilical cord cells compatible with a fetus, wherein the cells
5 express a gene product; and

b) exposing the fetus to the cells of step (a), wherein a chimeric allantois/umbilical cord capable of supplying the gene product to the fetus is created.

17. A method of evaluating the effect of test compounds on blood vessel formation comprising the steps of:

a) applying the test compound to a cultured
5 allantoic explant, and

b) observing the effect of the compound on vasculogenesis in the allantoic explant.

18. The method of claim 1 wherein the test compound
10 has a beneficial effect on blood vessel formation.

19. The method of claim 17 wherein the test compound has a detrimental effect on blood vessel formation.

20. The method of claim 17 wherein the test compound is a gene product.

21. A method of evaluating the effect of a test gene product on blood vessel formation comprising the step of:

- a) obtaining mesenchymal cells that express a specific test gene product;
- b) forming a transgenic allantois comprising the mesenchymal cells, wherein the test gene product is expressed; and
- c) observing the effect of the test gene product on vasculogenesis in the transgenic allantois.

22. The method of claim 20 wherein the test gene product is beneficial to vasculogenesis.

23. The method of claim 20 wherein the test gene product is detrimental to vasculogenesis.

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ABSTRACT OF THE DISCLOSURE

A method of fetal gene therapy is disclosed. In general, the method comprises the steps of identifying a fetus with a genetic defect, obtaining allantois/umbilical cord cells expressing a gene product that ameliorates the genetic defect, and exposing the fetus to the allantois/umbilical cord cells wherein a chimeric allantois is capable of supplying the gene product to the fetus is created. The present invention is also a method of examining the effect of test compounds on vasculogenesis and angiogenesis by observing the effect of the test compound on cultured allantoic explants.

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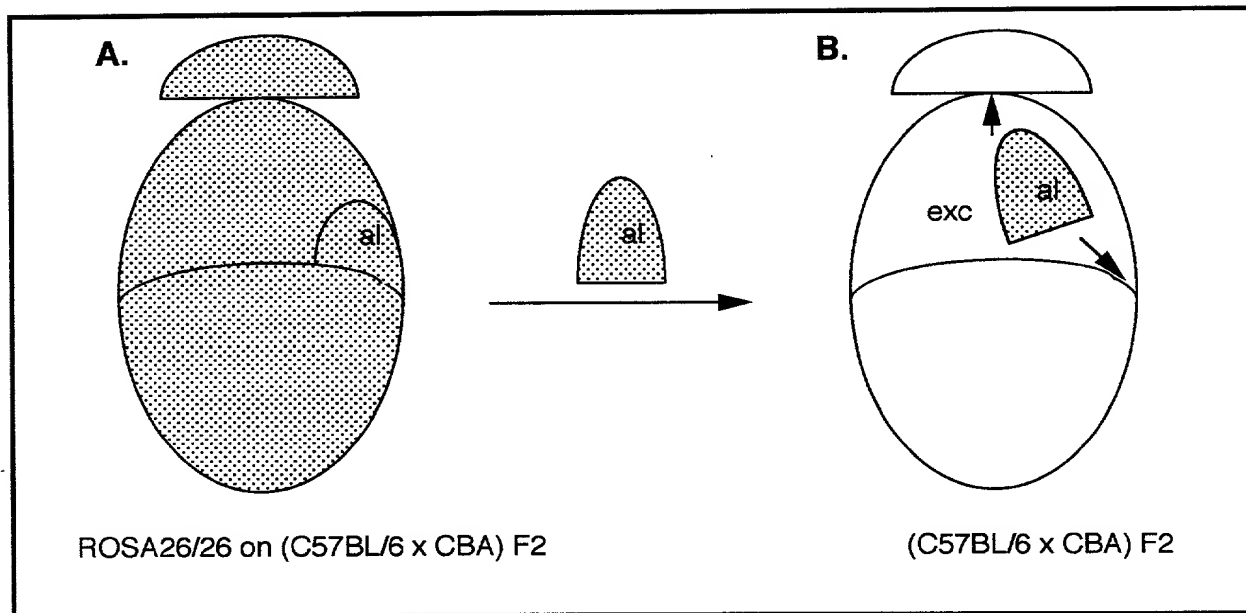
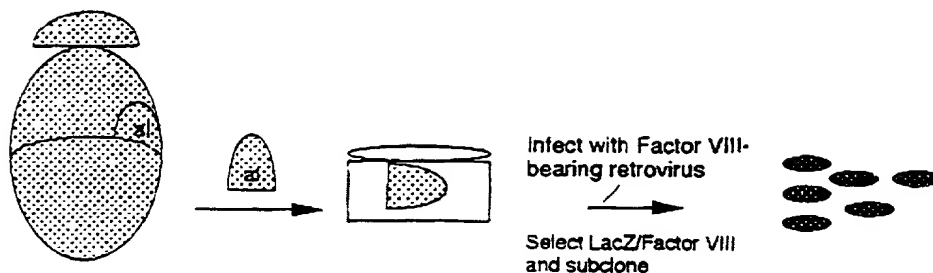


FIG. 1

1. Isolate LacZ-expressing allantois cells and infect with retrovirus carrying Factor VIII



2. Inject LacZ-Factor VIII-expressing allantois cells into Factor VIII-defective embryos

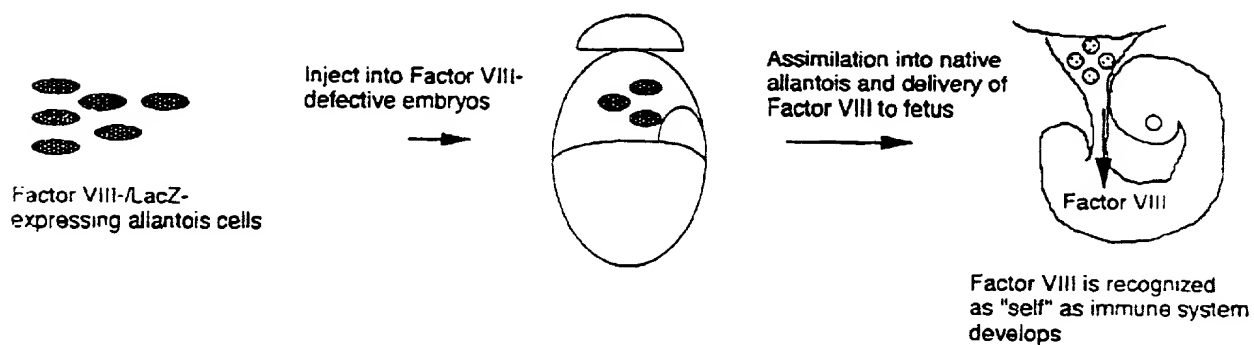


FIG. 2

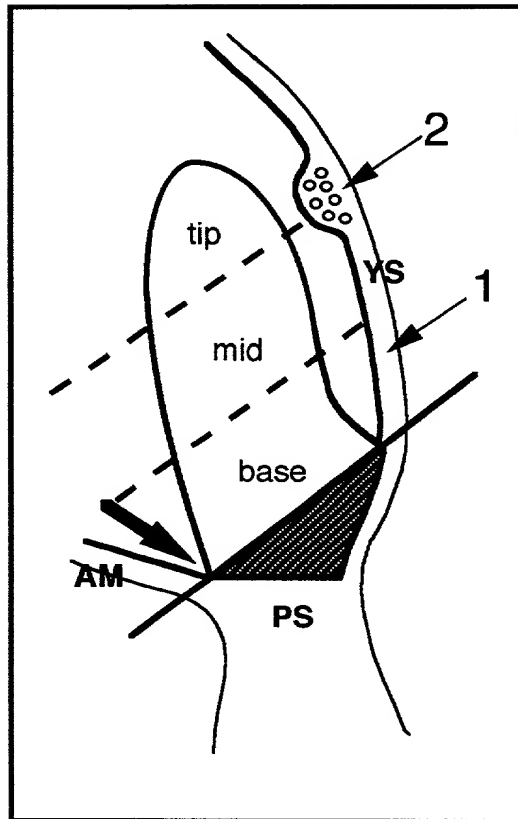


FIG. 3

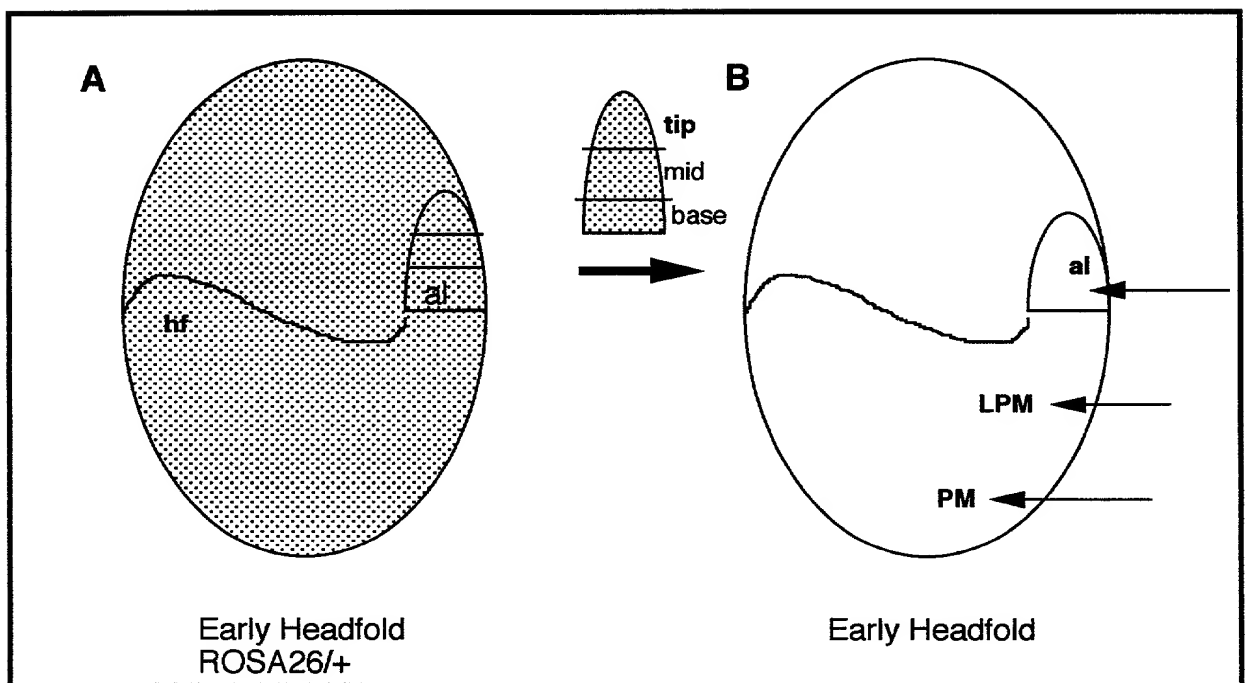


FIG. 4

Early Headfold
+A-






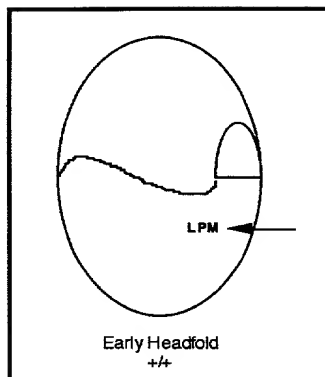
	75.0 - 99.9%
	50.0 - 74.9%
	10.0 - 49.9%
	1.0 - 9.9%
	<1.0%

FIG. 5



Summary of Grafts into the Primitive Streak at the Level of Prospective Lateral Plate Mesoderm				
	Orthotopic: Prospective Lateral Plate Mesoderm	Heterotopic: Allantois to Prospective Lateral Plate Mesoderm		
Tissue Grafted→ Tissue Colonized ↓	LPM	Base	Mid-Portion	Tip
Lateral Plate Mesoderm	+			
Intermediate Mesoderm				
Somites				
Neural Tube				
Capillaries				
Endoderm of Future Coelom				
Surface Ectoderm				
Endothelium of Intersegmental Vessels				
Endothelium of Aorta				
Mesenchyme Adj to Dorsal Aorta				
Umbilical Endothelium at All/Am Junction				
No Chimeras with Unincorporated Donor Cells		0	5	2
Initial Stage of Host Embryo	Headfold, 2-3	Headfold	Headfold, 3,4	Headfold
Final No. Somite Pairs	11-15	10-12	10-16	11-16
Total Number of Incorporated Transgenic Cells	1925	2789	2769	995
Number Chimeras (% of Total Injected)	10 (52.6%)	4 (23.5%)	12 (54.4%)	5 (27.8%)

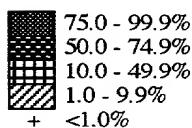
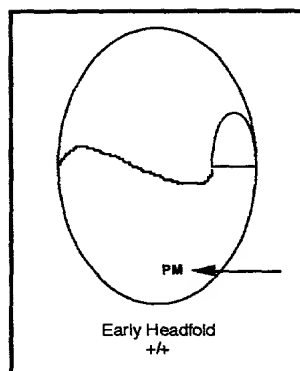


FIG. 6



Summary of Transplants into the Primitive Streak at the Level of Prospective Paraxial Mesoderm				
	Orthotopic: Prospective Paraxial Mesoderm	Heterotopic: Allantois to Prospective Paraxial Mesoderm		
Tissue Grafted→ Tissue Colonized ↓	PM	Base	Mid-Portion	Tip
Somites	75.0 - 99.9%			
Pre-Somitic Mesoderm	50.0 - 74.9%			
Capillaries (in neur ectoderm)	10.0 - 49.9%			
Neural Tube	1.0 - 9.9%			
Notochordal Plate	<1.0%			
Surface Ectoderm	+			
Endothelium of Intersegmental Vessels		75.0 - 99.9%	50.0 - 74.9%	
Endothelium of Dorsal Aorta		75.0 - 99.9%	50.0 - 74.9%	75.0 - 99.9%
Mesenchyme Adj to Dorsal Aorta		75.0 - 99.9%	50.0 - 74.9%	75.0 - 99.9%
No Chimeras with Unincorporated Donor Cells	1	0	4	1
Initial Stage of Host Embryos	Headfold, 3-4	Headfold	Headfold, 1	Headfold
Final Number Somite Pairs	10-14	9-11	8-13	11-13
Total Number of Incorporated Transgenic Cells	1021	136	1060	340
Number Chimeras (% Total Injected)	7 (33.3%)	2 (10.5%)	6 (28.6%)	4 (30.8%)

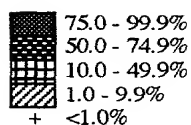


FIG. 7

FIG. 8

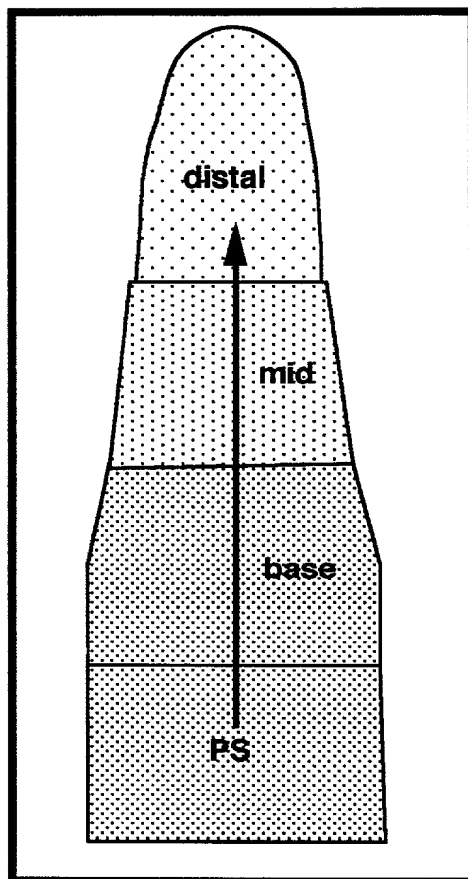


FIG. 9

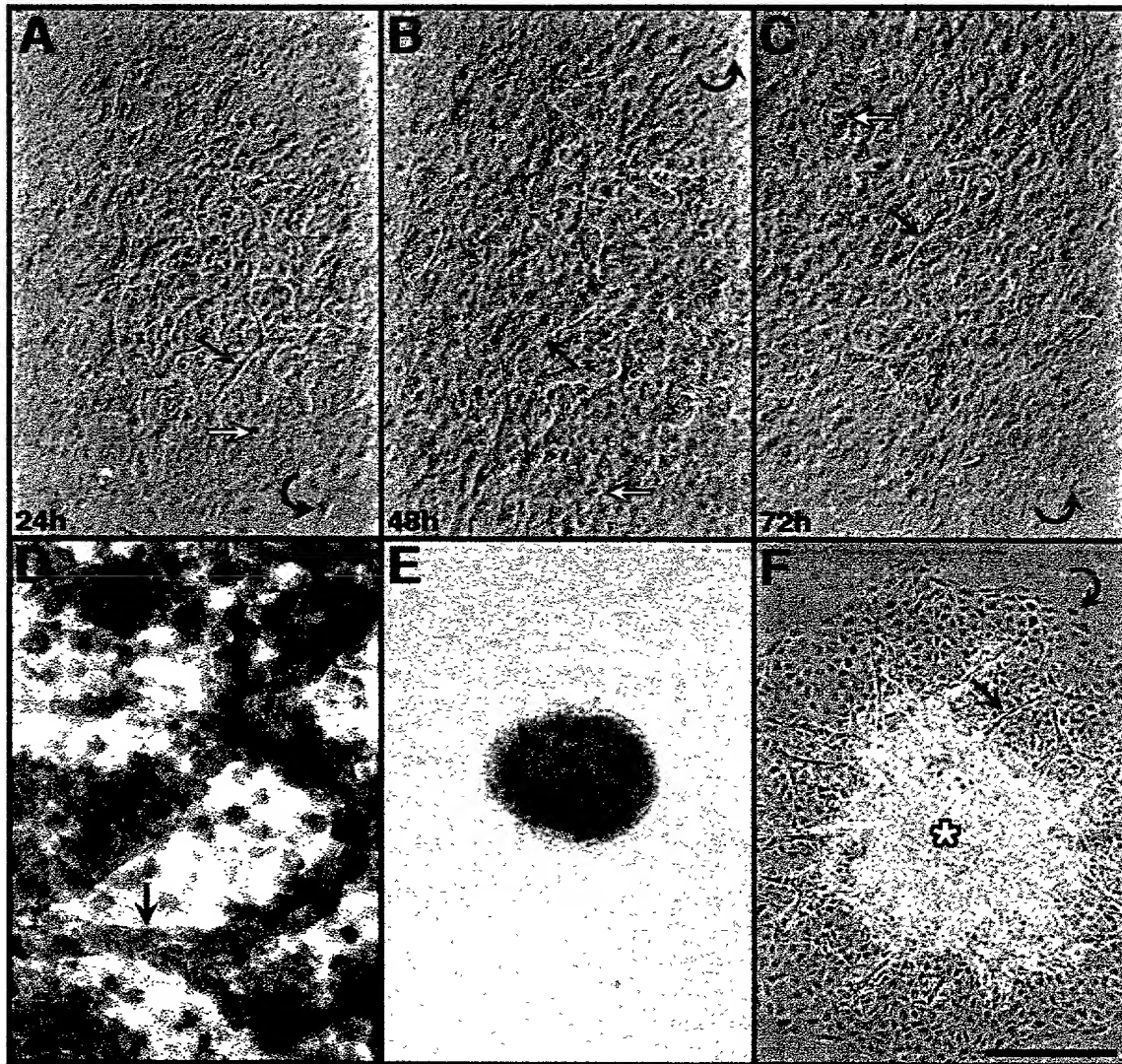


FIG. 10

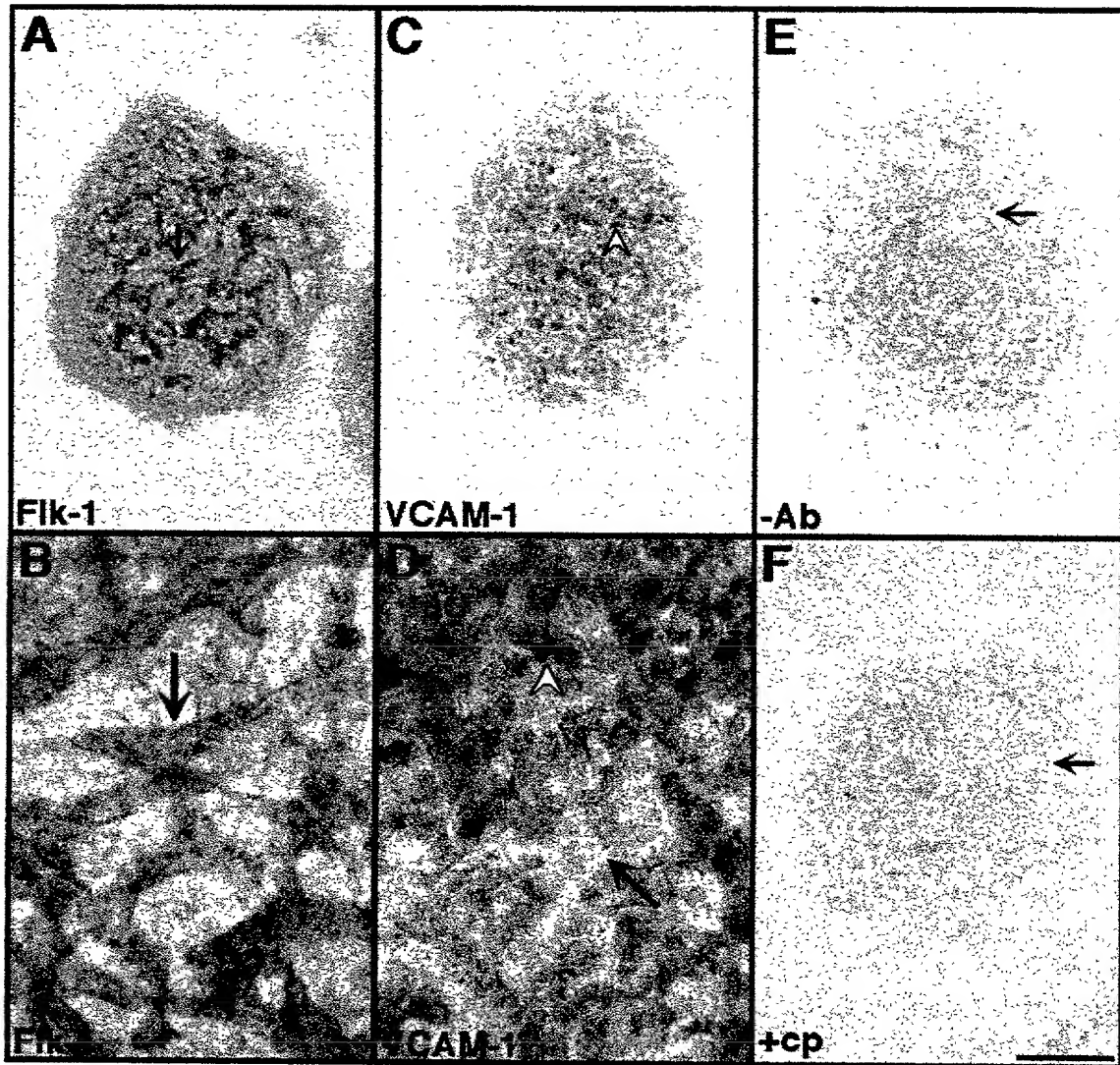


FIG. 11

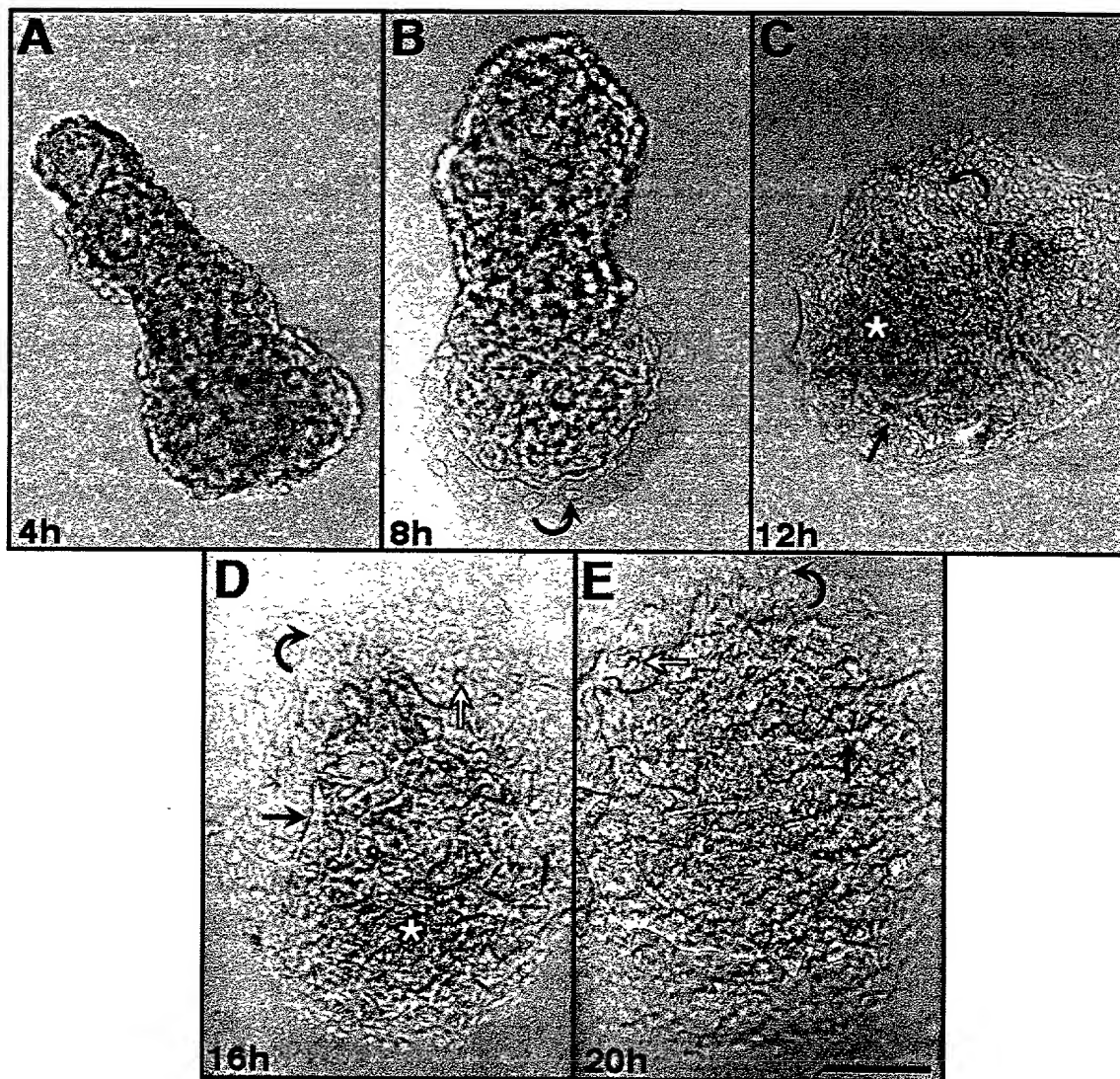


FIG. 12

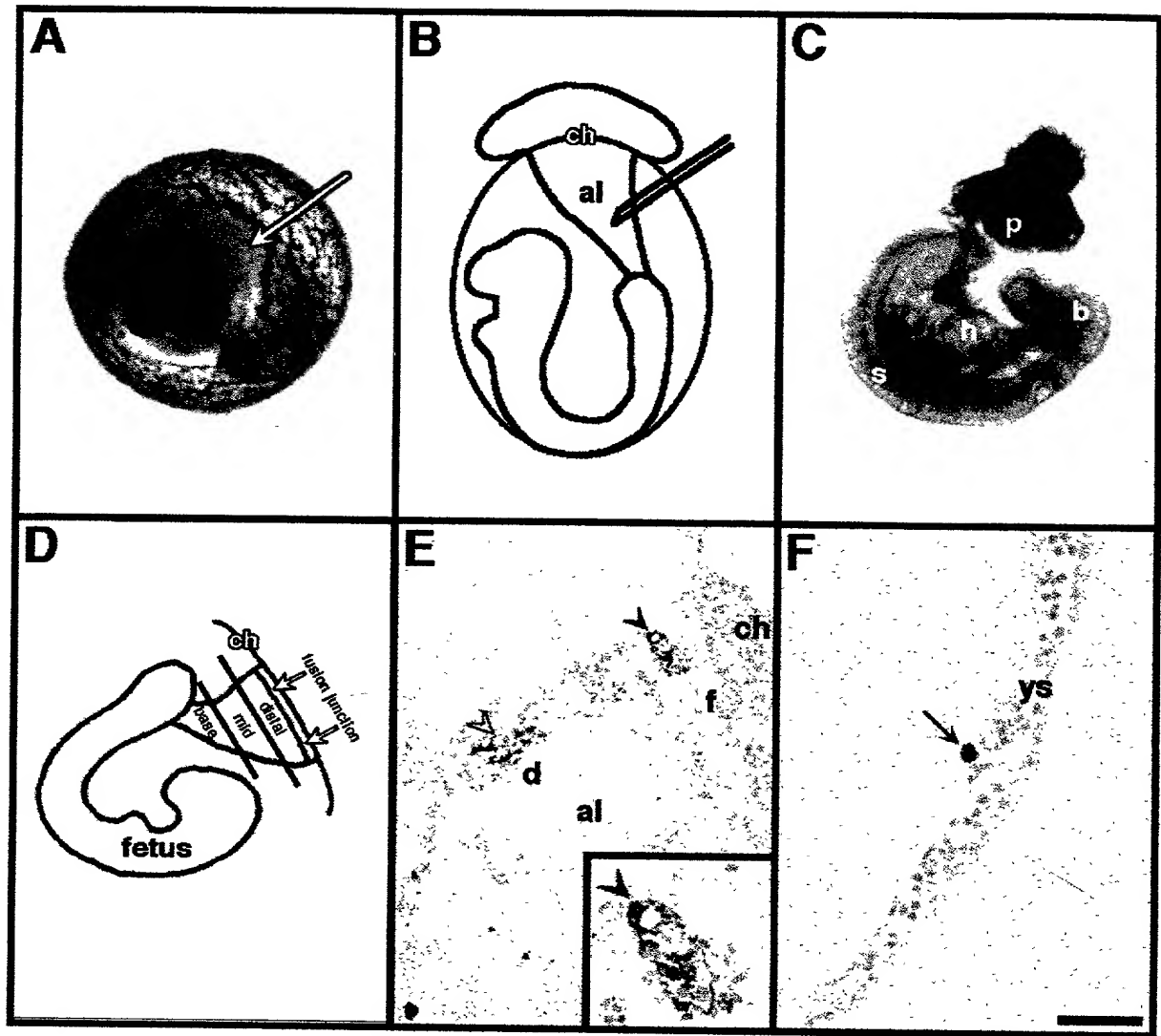


FIG. 13

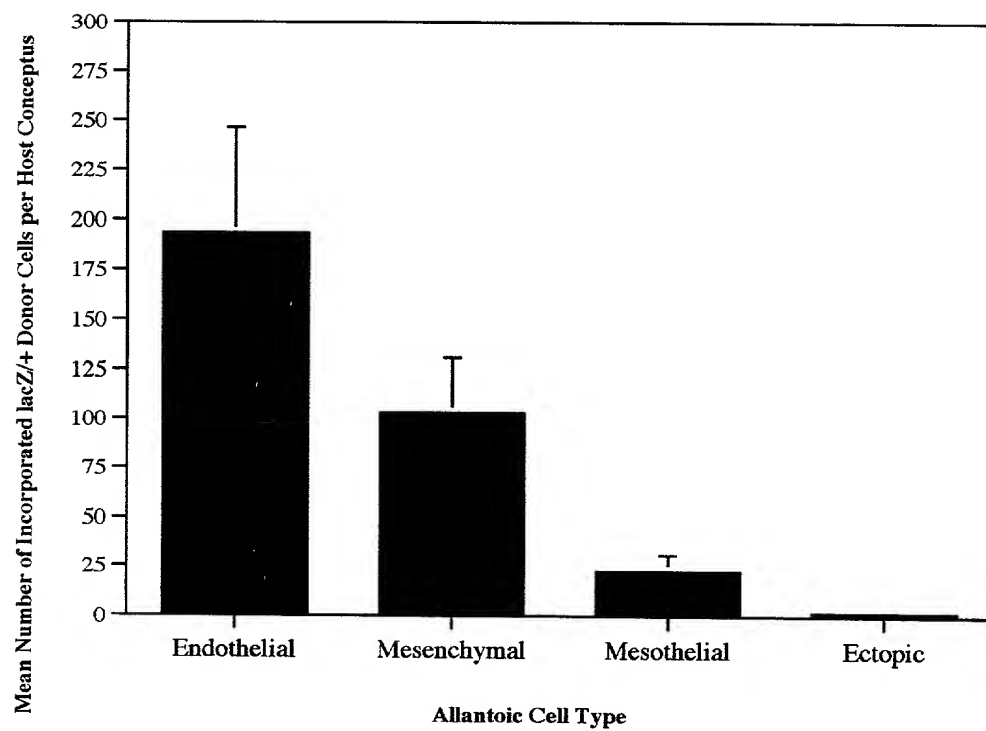


FIG. 14

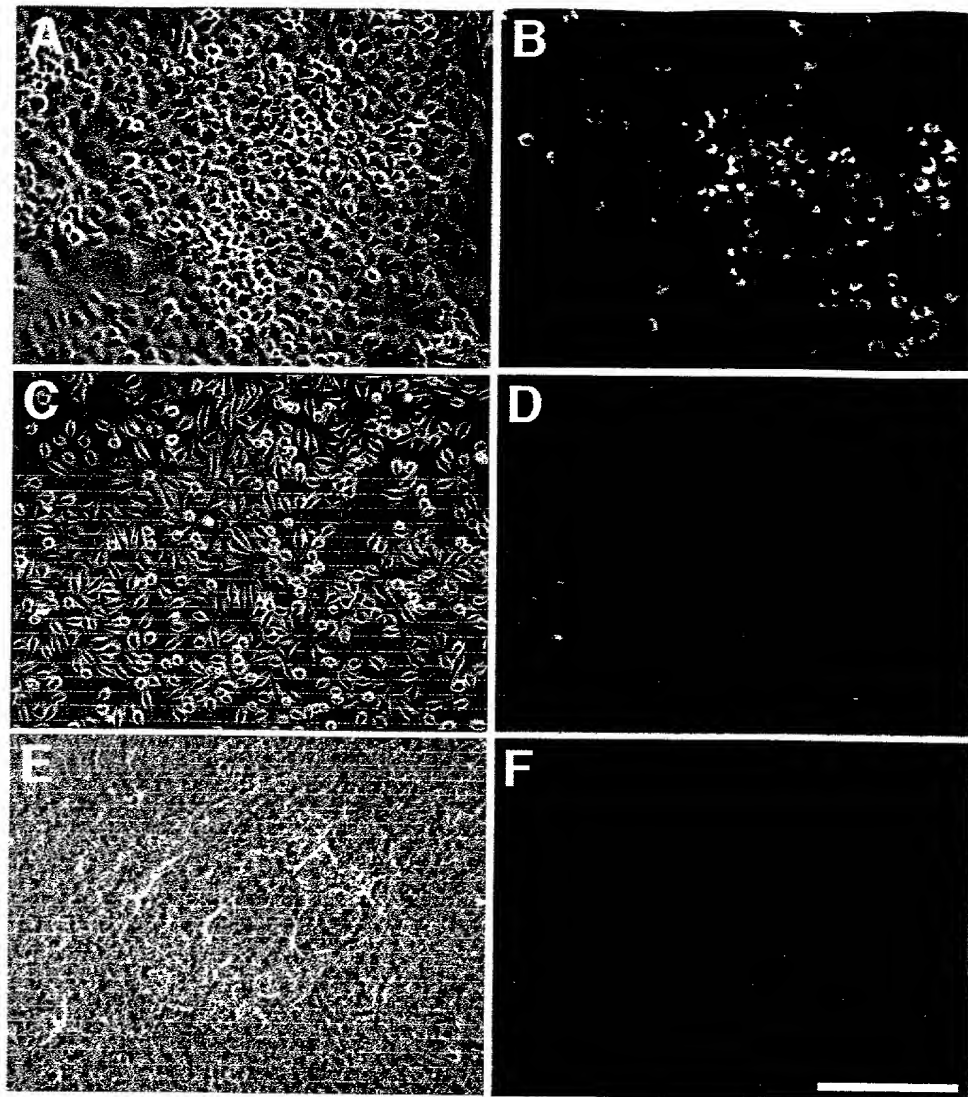


FIG. 15

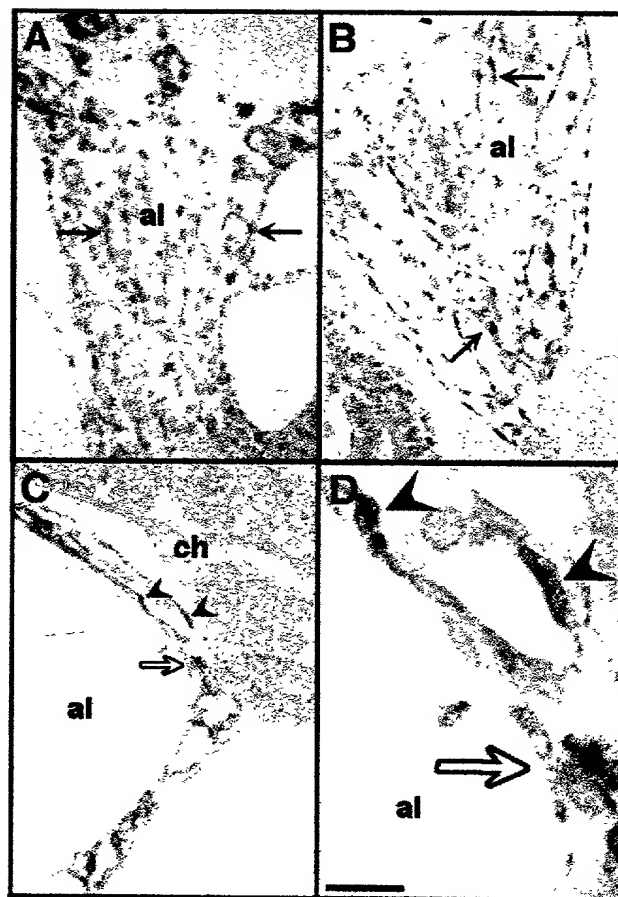


FIG. 16

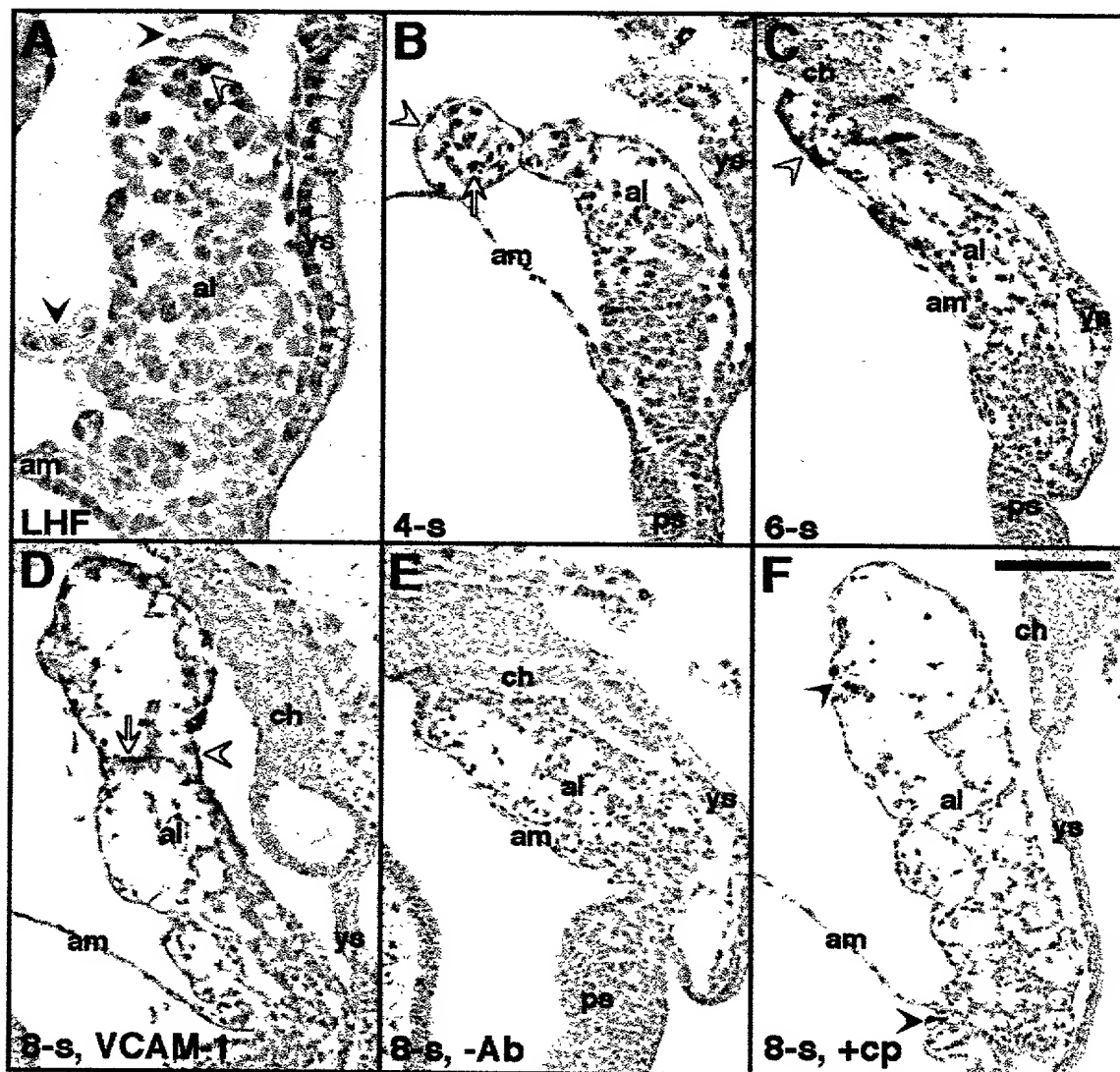


FIG. 17

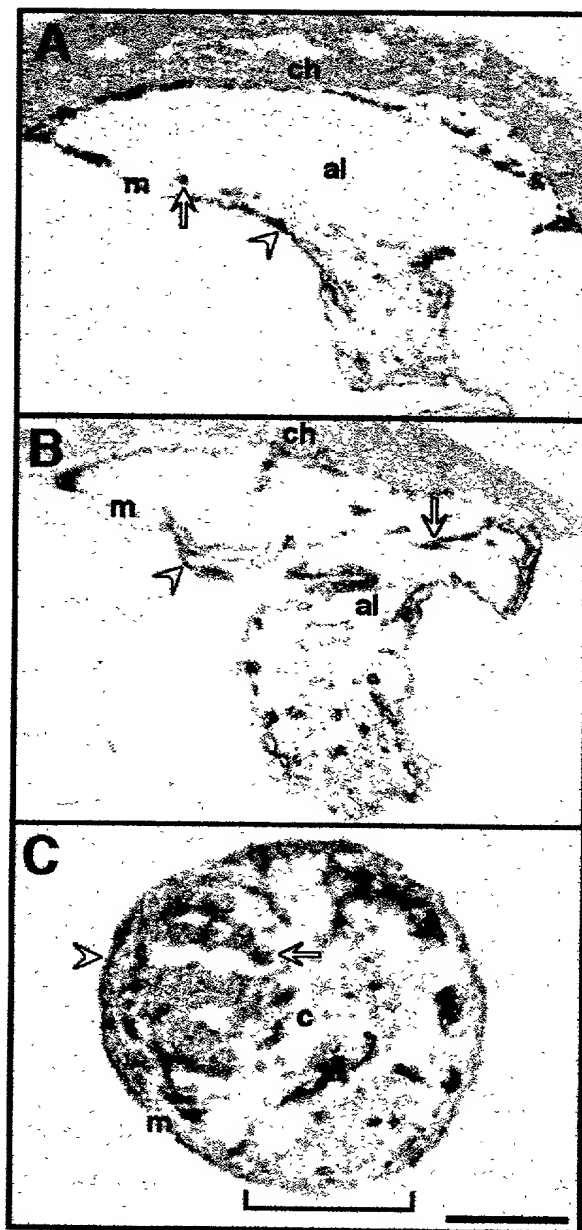


FIG. 18

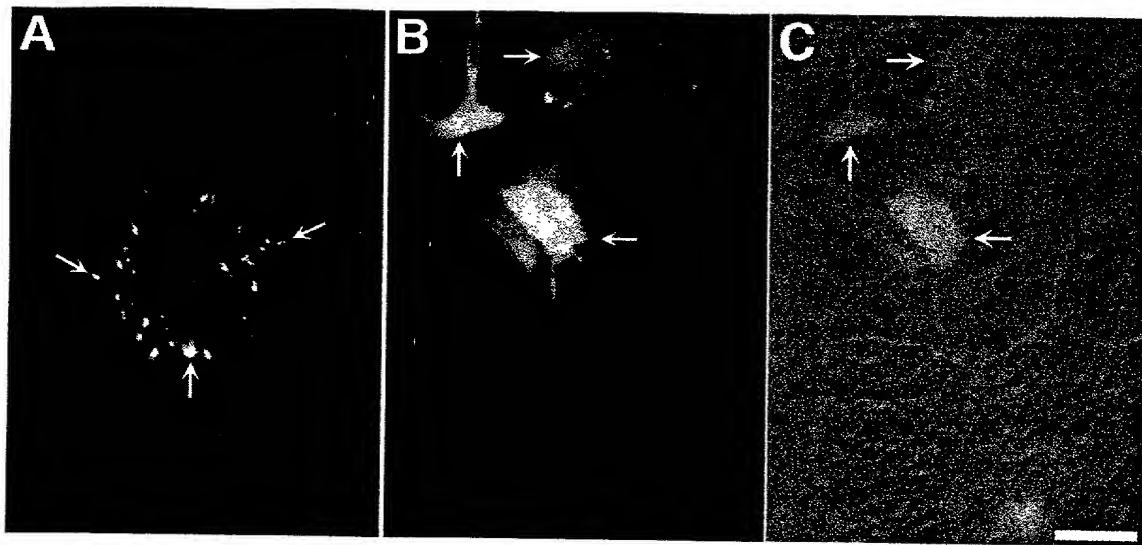


FIG. 19

Please type a plus sign (+) inside this box ☐

0010/PTO Rev. 6/95 U.S. Department of Commerce Patent and Trademark Office DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION <input checked="" type="checkbox"/> Declaration Submitted with Initial Filing OR <input type="checkbox"/> Declaration Submitted after Initial Filing	Attorney Docket Number	960296.95912
	First Named Inventor	Karen M. Downs
	COMPLETE IF KNOWN	
	Application Number	
	Filing Date	June 18, 1999
	Group Art Unit	
	Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

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(Title of the Invention)

the specification of which

☒ is attached hereto

OR

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Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES NO	
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☐ Additional foreign applications numbers are listed on a supplemental priority sheet attached hereto:

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Application Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.
60/015,066 60/118,764	April 9, 1996 February 5, 1999	

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DECLARATION

Page 2

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U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
08/838,384		April 8, 1997	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto

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Name of Sole or First Inventor: A petition has been filed for this unsigned inventor

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						Applicant Authority	

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